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# Differential Abundance Analyses of Human Microbiota in Parkinson's Disease


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# Differential abundance analyses of human microbiota in Parkinson's disease

Velma T. E. Aho

ACADEMIC DISSERTATION

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*La science, mon garçon, est faite d'erreurs,*
*mais d'erreurs qu'il est bon de commettre,*
*car elles mènent peu à peu à la vérité.*

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*Tiede hairahtuu, poikaseni,*
*mutta hairahdukset ovat hyödyllisiä,*
*sillä ne johdattavat vähitellen totuuteen.*

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*Science, my lad, has been built upon many errors;*
*but they are errors which it was good to fall into,*
*for they led to the truth.*

Jules Verne: Voyage au Centre de la Terre (1864)
Suomenkielinen käännös Arvo Airio (1917)
English translation Frederick Amadeus Malleson (1877)

# Abstract

Parkinson's disease is the second most common neurodegenerative disease in the world, and in spite of decades of research, the cause of the non-familial form of the disease is not known. There are currently no medications to slow down the progression of the disease nor good biomarkers for early diagnosis, even though the earliest non-motor symptoms can appear years or even decades before the onset of motor symptoms. The microbial inhabitants of the human body have recently been implicated in various medical conditions, including neurodegenerative disorders. They could offer new insight into the pathogenesis of Parkinson's disease, particularly since a microbial agent has long been suspected to play a part in the process. A typical question in studies surveying human microbiota is which specific microbial taxa differ between groups of interest, such as patients with a disease and control subjects. There are many statistical tools for performing these analyses, also known as differential abundance comparisons.

The aim of my doctoral thesis was to explore the potential associations of Parkinson's disease and human microbiota, particularly of the mouth, the nose and the gut, with an additional focus on the statistical tools used for comparing differentially abundant bacterial taxa. All four publications included in my thesis were based on samples from the same subjects: 76 patients with Parkinson's disease and 76 control subjects with no signs of parkinsonism. The studies also used the same methodology, 16S rRNA gene amplicon sequencing, to compare bacteria from oral and nasal swab samples and fecal samples of these subjects. Over the course of the four publications and in a previously unpublished analysis, I compared differentially abundant taxa with six tools: Metastats, LEfSe, metagenomeSeq, DESeq2, ANCOM, and an approach based on random forests.

Our results suggested that the bacterial communities of the gut and the mouth differ between Parkinson's patients and control subjects, with statistically significant differences in beta diversity and in the abundances of several bacterial taxa. Differences in gut microbiota could also be detected at a follow-up time point with samples collected two years after the initial sampling. Additionally, there were differences between the gut bacteria of Parkinson's patients with and without irritable bowel syndrome -like symptoms. For nasal bacteria, there were no differences between the patient and control groups in diversity nor the amounts of specific bacteria.

Regarding the differential abundance analyses, comparing gut bacteria of patients and controls from the same samples with six different tools highlighted the wide variation in the lists of significant results, which often did not overlap except for a handful of taxa. While a few benchmarking studies have previously contrasted some of the tools, there is a definite need for further standardized testing to guide researchers in choosing between them. Despite these discrepancies, all tools tested in this thesis supported Parkinson's patients having a decreased abundance of the family *Prevotellaceae* in their gut. This difference in abundance could also be detected at the follow-up time point. As several other research groups have reported seeing a decrease in *Prevotellaceae* after our pilot publication, it is emerging as one of the key changes in microbiota associated with Parkinson's disease.

# Abstrakti

Parkinsonin tauti on maailman toiseksi yleisin neurodegeneratiivinen sairaus, ja vuosikymmenten tutkimustyöstä huolimatta taudin ei-perinnöllisen muodon syy on edelleen arvoitus. Toistaiseksi ei ole olemassa lääkitystä, joka hidastaisi taudin etenemistä, eikä hyviä biomarkkereita taudin varhaiseen diagnoosiin, vaikka ensimmäiset ei-motoriset oireet saattavat alkaa vuosia tai jopa vuosikymmeniä ennen motorisia oireita. Ihmisruumiin mikrobiasukkaat on viime aikoina yhdistetty moniin tauteihin ja terveysongelmiin, mukaanlukien neurodegeneratiiviset sairaudet. Mikrobit voisivat tarjota uusia näkemyksiä Parkinsonin taudin patogeneesiin liittyen, etenkin kun niiden on jo pidempään arveltu vaikuttavan tautiprosessiin. Tyypillinen kysymys ihmisen mikrobistoa tutkittaessa on, mitkä tietyt taksonit eroavat vertailtavien ryhmien, esimerkiksi potilaiden ja verrokkihenkilöiden välillä. Mikrobien toisistaan poikkeavien määrien (englanniksi *differential abundance*) tilastolliseen vertailuun on olemassa monia eri työkaluja.

Väitöskirjani tavoite oli selvittää Parkinsonin taudin ja ihmisen suun, nenän ja suoliston mikrobien mahdollisia yhteyksiä sekä tarkastella samalla bakteerien määrien vertailuun käytettäviä työkaluja. Kaikki neljä julkaisuani perustuvat näytteisiin, jotka on kerätty samoilta koehenkilöiltä: 76 Parkinsonin tautia sairastavalta potilaalta sekä 76 verrokilta, joilla ei esiinny parkinsonismia. Julkaisuissa on käytetty samaa menetelmää, 16S rRNA-geenin amplikonisekvensointia, suu-, nenä- sekä ulostenäytteiden bakteerien määrittämiseen. Neljän julkaisun sekä ennen julkaisemattoman analyysin myötä vertailin ryhmien välillä poikkeavia bakteereja yhteensä kuudella eri työkalulla (Metastats, LEfSe, metagenomeSeq, DESeq2, ANCOM, sekä random forests).

Tulostemme perusteella Parkinsonin tautia sairastavien potilaiden ja verrokkihenkilöiden suoliston ja suun bakteeriyhteisöt poikkeavat toisistaan; ryhmien välillä oli tilastollisesti merkitsevä ero beta-diversiteetissä sekä useiden bakteeritaksonien määrissä. Suolistobakteeriyhteisöjen väliset erot voitiin myös havaita seurantanäytteissä, jotka oli kerätty kaksi vuotta ensimmäisen näytteenoton jälkeen. Lisäksi havaitsimme eroja suolistomikrobistossa, kun potilaat jaettiin luokkiin sen mukaan, oliko heillä ärtyvän suolen oireyhtymää muistuttavia oireita vai ei. Nenän bakteerien osalta emme löytäneet eroja diversiteetissä tai tiettyjen bakteerien määrissä potilaiden ja verrokkien välillä.

Mitä tulee bakteerien määrien tilastollisiin vertailuihin, potilaiden ja verrokkihenkilöiden suoliston bakteerien analyysit kuudella eri työkalulla korostivat eroja näiden työkalujen antamien merkitsevästi eroavien taksonien listoissa, joissa oli yleensä hyvin harvoja samoja bakteereja. Muutamat aiemmat julkaisut ovat vertailleet osaa näistä työkaluista, mutta olisi tarpeen tehdä laajempia, standardisoituja menetelmävertailuja, jotta tutkijoiden olisi helpompi valita, mitä työkalua käyttää. Eroavaisuuksistaan huolimatta kaikki tässä väitöskirjassa testatut työkalut tukivat havaintoa, että Parkinsonin tautia sairastavilla potilailla on suolistossaan vähemmän *Prevotellaceae*-heimon bakteereja kuin verrokeilla. Tämä heimo poikkesi ryhmien välillä myös seuranta-aikapisteessä. Koska vastaava eroavuus on ensimmäisen artikkelimme jälkeen havaittu useiden muiden tutkimusryhmien julkaisuissa, se on nousemassa yhdeksi keskeisistä Parkinsonin tautiin liittyvistä mikrobiston muutoksista.

# List of original articles

This thesis is based on the following original publications:

I. Scheperjans F, Aho V, Pereira PAB, Koskinen K, Paulin L, Pekkonen E, Haapaniemi E, Kaakkola S, Eerola-Rautio J, Pohja M, Kinnunen E, Murros K, Auvinen P (2015): Gut microbiota are associated with Parkinson's disease and clinical phenotype. Movement Disorders, 30(3): 350-358. DOI: 10.1002/mds.26069.

II. Pereira PAB*, Aho VTE*, Paulin L, Pekkonen E, Auvinen P, Scheperjans F (2017): Oral and nasal microbiota in Parkinson's disease. Parkinsonism and Related Disorders, 38: 61-67. DOI: 10.1016/j.parkreldis.2017.02.026. *equal contributors

III. Mertsalmi T, Aho VTE, Pereira PAB, Paulin L, Pekkonen E, Auvinen P, Scheperjans F (2017): More than constipation – Bowel symptoms in Parkinson's disease and their connection to gut microbiota. European Journal of Neurology, 24: 1375–1383. DOI: 10.1111/ene.13398.

IV. Aho VTE, Pereira PAB, Voutilainen S, Paulin L, Pekkonen E, Auvinen P, Scheperjans F: Gut microbiota in Parkinson's disease: Temporal stability and relations to disease progression. *Submitted manuscript*.

The publications are referred to in the text by their Roman numerals.

**Author's contributions:**

I. VTEA assisted in the bioinformatic sequence analysis, contributed to the design of the statistical analyses, performed the initial statistical analyses, contributed to interpreting the results, and wrote the manuscript together with co-authors.

II. VTEA performed the bioinformatic sequence analysis and the statistical analyses for nasal microbiota, wrote the first draft for the corresponding sections of the article, contributed to the interpretation of oral microbiota results, and wrote the manuscript together with co-authors.

III. VTEA performed the statistical analyses of gut microbiota data, wrote the corresponding sections, and participated in writing the rest of the manuscript together with co-authors.

IV. VTEA performed the bioinformatic sequence analysis and statistical data analyses, participated in interpreting the results, wrote the first draft of the manuscript, and prepared the finished manuscript together with co-authors.

# Contents

# Abbreviations

**AD** Alzheimer's disease

**ASD** autism spectrum disorder

**CNS** central nervous system

**COMT** catechol-O-methyl transferase

**FDR** false discovery rate

**FFQ** food frequency questionnaire

**GABA** gamma-aminobutyric acid

**GF** germ-free

**GI** gastrointestinal

**GLM** generalized linear model

**IBS** irritable bowel syndrome

**LED** levodopa equivalent dose

**LFC** logarithmic fold change

**LP** Lewy pathology

**NMDS** non-metric multidimensional scaling

**NMSS** non-motor symptoms scale

**OTU** operational taxonomic unit

**PD** Parkinson's disease

**qPCR** quantitative PCR

**RBD** rapid eye movement (REM) sleep behavior disorder

**RBDSQ** REM sleep behavior disorder screening questionnaire

**RNA-seq** RNA sequencing

**rRNA** ribosomal ribonucleic acid

**SCFA** short-chain fatty acid

**SCS-PD** sialorrhea clinical scale for Parkinson's disease

**SDQ** swallowing disturbance questionnaire

**TIA** transient ischemic attack

**UPDRS** unified Parkinson's disease rating scale

**ZIG** zero-inflated Gaussian

# 1 Introduction

## 1.1 The human microbiome

Human beings are never truly alone. Our bodies contain countless cells that are not our own: archaea, bacteria, and microscopic eukaryotes, including fungi. Even greater in number are viruses, which target both human cells and our microbial inhabitants. Collectively, all these organisms can be referred to as *microbiota*, while the ensemble of the organisms and their specific environment, such as cells and metabolites of the host body, make up a *microbiome* (Young, 2017). Although the terms refer to microbes of all kingdoms, the focus of human microbiome research so far has been on bacteria due to their clinical significance, and because of the technical challenges in characterizing other types of microbes.

Traditional culture-based methods only capture a small subset of all microorganisms. Woese and colleagues pioneered using the 16S ribosomal RNA (rRNA) gene for microbial identification (Woese and Fox, 1977). Culture-independent methods that build on their work and target the 16S rRNA gene, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and Sanger sequencing, have been used in microbial ecology for decades, mainly in an environmental context (Grice and Segre, 2012). A more recent technology, DNA microarrays, offered much higher throughput, but was still limited to detecting previously known target microbes (Cardenas and Tiedje, 2008). In the early 2000s, the advent of high-throughput next generation sequencing technologies led to growing interest in exploring complex microbial communities, including those inhabiting humans.

Currently, the most popular method for microbial community analysis is targeted sequencing of a specific marker gene, also known as amplicon sequencing: the target gene is amplified with PCR, sequenced, and the resulting sequence reads classified bioinformatically using a reference database. The 16S rRNA gene is the most common target for this approach for the same reasons that made it the marker of choice for earlier methods: it has conserved sequences suitable for targeting with universal primers and variable regions enabling microbial classification (Tringe and Hugenholtz, 2008; Větrovský and Baldrian, 2013; Woese and Fox, 1977). Analogous analyses of fungal communities, sometimes termed the *mycobiome*, typically use internal transcribed spacer sequences (ITS), or sometimes the 18S rRNA gene, which is more highly conserved and therefore offers less taxonomic information (Cui et al., 2013; Knight et al., 2018). More comprehensive approaches, such as sequencing all DNA (shotgun metagenomics) or all RNA (metatranscriptomics) of an environmental sample, have also gained popularity, allowing insight into the functional capabilities and actual activities of the microbes (Knight et al., 2018).

Thanks to undertakings such as the Human Microbiome Project, the microbial communities residing in different parts of the human body have been characterized extensively, revealing significant interpersonal variation and a few key taxa that dominate specific body locations (Grice and Segre, 2012; Human Microbiome Project Consortium, 2012; Turnbaugh et al., 2007). Experimental studies in germ-free (GF) animals have shown that microbiota are essential to normal maturation of the immune system (Chung et al., 2012; Erny et al., 2015), and perhaps even to brain development (Dinan and Cryan, 2017).

This has led to a shift in thinking where instead of simple relationships of one particular pathogen causing a disease, the human body should be seen as a complex ecological entity full of interactions (Vayssier-Taussat et al., 2014; Vonaesch et al., 2018).

Questions about microbial links to various diseases and medical conditions have always been at the forefront of human microbiome research. The field is starting to move from purely descriptive studies towards ones that will offer more insight into mechanisms and hopefully lead to future diagnostic and therapeutic innovations (Gilbert et al., 2018). With the realization of the overall importance of microbiota to our health, research has expanded from diseases traditionally linked to pathogenic microbes to those where the association is not as intuitive, including metabolic syndrome and obesity, and neurodegenerative diseases (Young, 2017). In addition to the microbial communities of the three anatomic locations that are the focus of this thesis – the nose, the mouth and the gut – there have been studies concerning various other body sites. The most commonly studied ones are probably skin (Byrd et al., 2018) and reproductive organs, particularly vaginal microbiota (Anahtar et al., 2018). Among the other sites that have been characterized are ears (Kalcioglu et al., 2018; Minami et al., 2017), ocular surfaces (Kugadas and Gadjeva, 2016), lower airways (Aho et al., 2015), the bladder (Thomas-White et al., 2016), and bile ducts (Pereira et al., 2017).

### 1.1.1 Nasal microbiota

The nasal epithelium is an important contact surface between the external environment and the body (de Steenhuijsen Piters et al., 2015). Sinonasal microbiota are typically studied using either swab sampling or sinus lavage (Bassiouni et al., 2015; Jervis Bardy and Psaltis, 2016; Kim et al., 2015a). A third alternative could be mucosal tissue biopsies, but they offer no significant advantage, since they are more invasive and produce results similar to swabs (Bassiouni et al., 2015; Jervis Bardy and Psaltis, 2016; Kim et al., 2015a). In swab sampling, the choice of the specific anatomical site is an important consideration; there is no consensus for the best approach, and communities inhabiting different microenvironments of the nasal cavity are not identical, although interpersonal variation is greater than intrapersonal (Ramakrishnan et al., 2016; Ramakrishnan et al., 2017; Yan et al., 2013).

The nasal microbial community reaches its mature adult state during puberty, and consists mostly of *Actinobacteria* (such as *Corynebacterium* and *Propionibacterium*) and *Firmicutes* (particularly *Staphylococcus*) (Bomar et al., 2018; Brugger et al., 2016; de Steenhuijsen Piters et al., 2015). A recent study using a propidium monoazide dye approach to specifically capture viable cells found the majority of such cells to represent the *Staphylococcaceae* and *Corynebacteriaceae* families (Lu et al., 2018). Many clinically important species, for example *Staphylococcus aureus* and *Streptococcus pneumoniae*, are typical colonizers of nasal passages (Bomar et al., 2018; Brugger et al., 2016). Although these bacteria can be pathogenic, more often they are a harmless part of the community; such common microbes with pathogenic potential have been called pathobionts (Brugger et al., 2016; Mazmanian et al., 2008).

A study with mono- and dizygotic twins suggested that nasal microbial communities are not strongly dependent on host genetics, since the nasal microbiota of monozygotic twins were not significantly more similar than those of dizygotic twin pairs (Liu et al., 2015). Environmental effects may be more important to shaping nasal microbial communities. Cigarette smoking, a potentially important environmental exposure, was reported as having a significant effect on nasal microbiota in one study (Ramakrishnan and Frank, 2015), but none at all in another (Yu et al., 2017). As for specific medical conditions, sinonasal microbiota have been mostly studied in the contexts of *S. aureus* carriage and chronic rhinosinusitis

(Wilson and Hamilos, 2014). Although there is no clear consensus regarding the changes related to rhinosinusitis, several studies have detected an increased abundance of *S. aureus*, as well as a reduced species richness and diversity (Ramakrishnan et al., 2016). In diseases not directly involving the nose, Johnson et al. explored the potential link of nasal microbiota and skin and soft tissue infections, which are commonly caused by *S. aureus*, finding that the bacterial communities of healthy controls' anterior nares contained more *Proteobacteria*, and that overall, there was an inverse correlation between the abundances of *S. aureus* and *Corynebacterium* (Johnson et al., 2014). The same inverse correlation has been reported by two other studies (Lemon et al., 2010; Yan et al., 2013).

### 1.1.2 Oral microbiota

The oral cavity is another important interaction surface between the body and the environment, and the entry point to the gastrointestinal (GI) system (Hall et al., 2016). Its physical environment changes during the human lifespan, from the toothless newborn, through primary and permanent dentition, to older age where periodontitis is particularly common possibly due to aging-related alterations in immune and inflammatory status (Feres et al., 2016; Mason et al., 2018). The oral cavity contains several differing microbial communities: for example, the microbiota of supra- and subgingival plaque, which resemble each other, are distinct from those of saliva, tongue, tonsils and throat (Hall et al., 2016; Segata et al., 2012; Simón-Soro et al., 2013). This means that the choice of sampling site is particularly important. Nevertheless, although it is unclear how well salivary microbiota capture clinically interesting changes in supra- and subgingival microbial communities, saliva is commonly used as a proxy for oral health due to its easy availability (Shi et al., 2018; Simón-Soro et al., 2013). It has been pointed out that the true spatial complexity of oral microbiota is inevitably lost when samples are collected and homogenized for sequencing-based analysis approaches (Mark Welch et al., 2016).

The expanded Human Oral Microbiome Database, a curated collection of oral bacterial 16S rRNA gene sequences and genomes, currently contains 770 species, 70% of which are either named or unnamed but cultivated, with the remaining 30% representing uncultivated phylotypes (expanded Human Oral Microbiome Database (eHOMD), 2018; Chen et al., 2010). This can be seen as one approximation for the number of bacterial species typically residing in the oral cavity. Overall, all oral niches contain many of the same genera, such as *Streptococcus* and *Veillonella* (phylum *Firmicutes), Rothia (Actinobacteria), Prevotella (Bacteroidetes)*, and *Fusobacterium (Fusobacteria)*, but at differing relative abundances (Hall et al., 2016; Mason et al., 2018; Segata et al., 2012; Simón-Soro et al., 2013). The composition of the oral microbial communities and the amount of temporal fluctuations in them are highly individualized (Hall et al., 2016). As with nasal microbiota, it seems that oral microbiota are shaped more by environmental factors than genetics (Shaw et al., 2017; Stahringer et al., 2012), although the effects of specific factors, such as geographic location, climate or diet, are still poorly understood (Li et al., 2014; Nasidze et al., 2009).

Oral microbes have been studied for a long time in traditional microbiology due to their importance for dental health, and modern microbiome research often focuses on the most common oral bacterial diseases, periodontitis and caries (Krishnan et al., 2017). However, there is a growing interest in potential links between oral microbiota and systemic diseases, such as rheumatoid arthritis (Potempa et al., 2017), cardiovascular diseases (Pietiäinen et al., 2018), and Alzheimer's disease (AD) (Fulop et al., 2018; Harding et al., 2017; Shoemark and Allen, 2015). Periodontitis is associated with a higher risk of AD (Chen et al., 2017; Pritchard et al., 2017), and there have been suggestions of an infectious origin for AD,

with hypothesized involvement of several different bacteria such as spirochetes and the oral pathogen *Porphyromonas gingivalis* (Fulop et al., 2018; Pritchard et al., 2017). There is some preliminary evidence from small studies for abnormal presence of oral bacteria in the brain (Emery et al., 2017) and elevated serum antibodies to oral pathogens (Sparks Stein et al., 2012) in AD patients. Although the picture is far from clear, a connection between AD and oral microbiota seems possible.

### 1.1.3 Gut microbiota

Out of all microbial communities in the human body, gut microbes are probably the one that has been studied the most up to date; between the years 2013 and 2017, there were nearly 13 000 publications concerning gut microbiota (Cani, 2018). Since obtaining samples from inside the GI tract requires invasive procedures, most of these studies use stool samples to get a snapshot view of the gut community (Cani, 2018; Marchesi et al., 2016). In fact, there is considerable variation along the GI tract, including between mucosal and luminal sites of the colon (Donaldson et al., 2016), and as could be expected, the bacteria in stool most closely resemble those in the distal lumen (Flynn et al., 2018). Although the large number of studies implies that the factors affecting an individual's gut microbial community and the microbes' role in health and disease have been characterized extensively, many key questions remain unanswered, and therapeutic breakthroughs are few and far between (Cani, 2018; Marchesi et al., 2016).

The first colonizers of an infant's gut originate from the mother. In addition to vaginal microbiota, which are missing from babies delivered by C-section, they include gut, oral, skin and breast milk derived taxa; some studies have suggested that the earliest colonization may take place *in utero*, but this has been contested by others (Greenhalgh et al., 2016; Perez-Munoz et al., 2017; Rodríguez et al., 2015; Sprockett et al., 2018). It was long assumed that a child's gut microbiome reaches an adult-like state fairly early, between 3-5 years of age (Rodríguez et al., 2015; Yatsunenko, 2012), although several recent studies have challenged this consensus, reporting differences between the gut microbial communities of children, adolescents and adults (Greenhalgh et al., 2016).

The gut bacterial communities of adults are dominated by two phyla: *Bacteroidetes* and *Firmicutes*, followed by *Actinobacteria* and *Proteobacteria*, and several less common phyla, such as *Fusobacteria* (Rajilić-Stojanović and de Vos, 2014; Segata et al., 2012). The main genera include *Alistipes, Bacteroides, Parabacteroides* and *Prevotella (Bacteroidetes); Lactobacillus, Streptococcus, Veillonella* and bacteria of the *Clostridia* clusters, such as *Blautia, Clostridium, Faecalibacterium* and *Ruminococcus (Firmicutes); Escherichia (Proteobacteria); Bifidobacterium (Actinobacteria)*; and *Fusobacterium (Fusobacteria)* (Chen et al., 2016a; Rajilić-Stojanović and de Vos, 2014; Segata et al., 2012). As with other microbial communities of the human body, there is a wide range of interindividual variability even in healthy subjects (Chen et al., 2016a; Costea et al., 2018; Rajilić-Stojanović and de Vos, 2014; Segata et al., 2012). On the other hand, a few key taxa are typically dominant, and these have been used to cluster the communities into categories, commonly known as enterotypes (Arumugam et al., 2011; Costea et al., 2018). Different studies have resulted in different optimal categorization results, but a proposed consensus includes three enterotypes, dominated by either *Bacteroides, Prevotella* or bacteria of the *Firmicutes* phylum (Costea et al., 2018).

The microbial community in a healthy adult's gut is quite stable over time (Faith et al., 2013; Flores et al., 2014; Voigt et al., 2015), although the amount of variability itself varies individually (Flores et al., 2014). Some perturbations, such as antibiotic treatment, may have

long-standing effects (Voigt et al., 2015). Many demographic and lifestyle factors have been associated with gut microbiota composition in multiple studies, the key ones including diet (Chen et al., 2016a; Claesson et al., 2012; David et al., 2014; Davis et al., 2017), medications (Forslund et al., 2015), age (Yatsunenko, 2012), and BMI (Chen et al., 2016a; Davis et al., 2017; Marchesi et al., 2016; Rothschild et al., 2018). There are major differences between the gut bacterial communities of subjects living in non-urban, non-Westernized societies and their urban counterparts; in these cases, unraveling the many variables affecting community composition is not simple (Fragiadakis et al., 2018; Martinez et al., 2015). An additional factor associated with gut microbiota profiles in stool samples is stool consistency, which can be considered a proxy for intestinal transit time (Vandeputte et al., 2016).

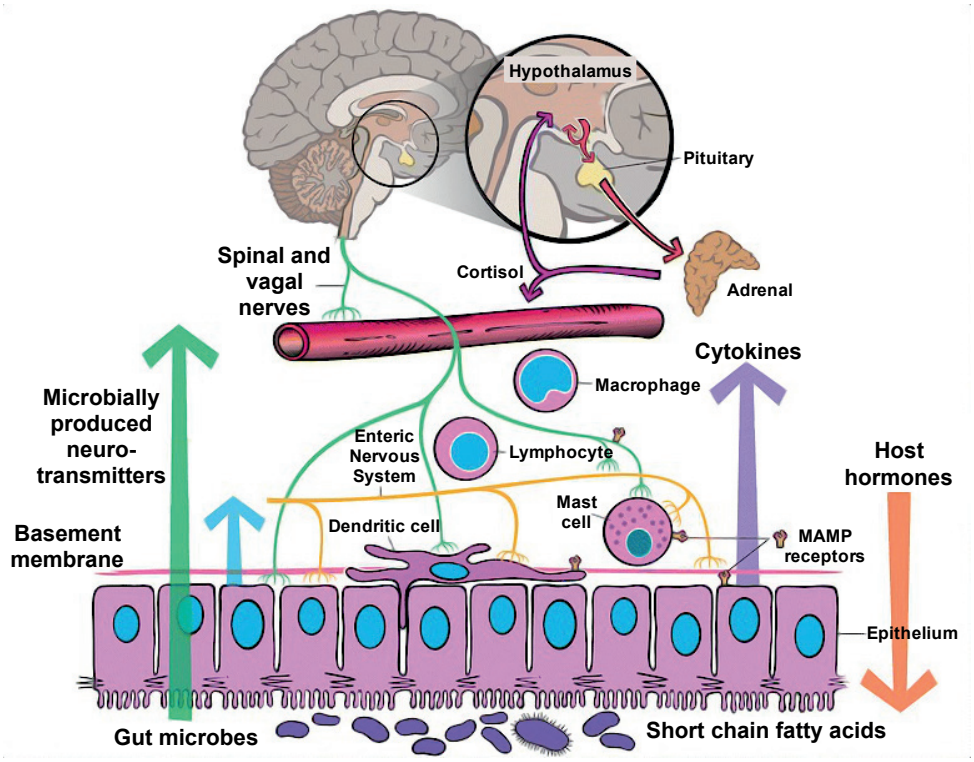
The human host's genotype also plays an important part in shaping the gut microbial community; studies have reported that both alpha diversity and some specific bacterial taxa are heritable, and that many immune-related genes are associated with microbiota composition (Goodrich et al., 2017). As a specific example of genetic effects, an increase in *Bifidobacterium* abundance has been associated with the SNP leading to a lactase non-persistence phenotype (Blekhman et al., 2015; Goodrich et al., 2017). On the other hand, one recent study concluded that overall, the role of the environment in determining an individual's microbiota seems greater than that of genetics (Rothschild et al., 2018).

Regarding gut microbiota in health and disease, early research often focused on directly GI-tract related medical conditions, such as *Clostridium difficile* infection (Jalanka et al., 2018), inflammatory bowel disease (Sheehan et al., 2015) and irritable bowel syndrome (IBS) (Bhattarai et al., 2017). The greatest single success of a gut microbiome related therapy up to date has been the treatment of recurrent *C. difficile* infection with fecal microbiota transplantation (Jalanka et al., 2018; Ooijevaar et al., 2018). Numerous studies have also explored probiotics ("live microorganisms that, when administered in adequate amounts, confer a health benefit on the host," Hill et al., 2014) and prebiotics ("a substrate that is selectively utilized by host microorganisms conferring a health benefit," Gibson et al., 2017) as potential treatment modalities. Probiotics in particular have been tested in various contexts, but while there are promising preliminary results, there is no consensus regarding strain selection and dose for any specific condition (Hooks et al., 2018; Kristensen et al., 2016; Rondanelli et al., 2017). Within the past decade, with the growing appreciation of the overall significance of gut microbiota, research has expanded beyond directly GI-connected topics and uncovered associations between gut microbiota and a variety of conditions, such as liver diseases (Chassaing et al., 2014), rheumatic diseases (Scher et al., 2016), and neurological diseases like autism spectrum disorder (ASD) (Hughes et al., 2018) and multiple sclerosis (Mirza and Mao-Draayer, 2017).

### 1.1.4 The microbiome–gut–brain axis

The rationale behind studying gut microbiota in the context of neurological diseases is based on the concept of the microbiome–gut–brain axis, the interaction between gut microbiota, the GI tract and the central nervous system (CNS) (Dinan and Cryan, 2017; Forsythe et al., 2016). The idea of a link between the gut and the brain is not new; the earliest scientific explorations of it can be traced to the late 19th and early 20th century (Aziz and Thompson, 1998). The anatomical connections between the gut and the brain, based on the vagal and spinal nerves, are well established (Aziz and Thompson, 1998; Forsythe et al., 2016). The vagus nerve is the longest cranial nerve, and in addition to providing parasympathetic innervation to the GI tract, it is involved in processes ranging from cardiovascular and respiratory regulation to modulating immune responses (Browning et al., 2017). The immune

Figure 1.1: Diagram of the proposed links between microbiota, the gut, and the brain.




MAMP: microbe-associated molecular pattern

Figure simplified from Figure 1 in Forsythe et al., 2016; used under the CC BY 4.0 licence.

and endocrine systems form another layer of communication between the gut and the brain (Forsythe et al., 2016). Although potential effects of gut microbes or their metabolites on the nervous system have been explored for decades, the current wave of microbiome–gut–brain axis research has evolved hand in hand with the DNA-based tools used in modern microbiome studies (Forsythe et al., 2016; Hooks et al., 2018).

The strongest evidence for the importance of the microbiome–gut–brain axis comes from animal experiments, typically with GF mice (Dinan and Cryan, 2017; Hooks et al., 2018). A particularly influential early study showed an altered stress response in GF mice which could be partly rescued with early-life introduction of *Bifidobacterium infantis* or fecal microbiota from specific pathogen free (SPF) mice (Sudo et al., 2004). Later research using similar approaches has further demonstrated that bacteria can affect both behavior and brain development in rodents (Dinan and Cryan, 2017; Forsythe et al., 2016; Hooks et al., 2018). Among the most cited publications (Hooks et al., 2018) are a study reporting that GF and SPF mice showed marked differences in motor and anxiety behavior and the expression of related genes, with early-life gut microbiota restoration rescuing the phenotype (Diaz Heijtz et al., 2011); one demonstrating that *Lactobacillus rhamnosus* can alleviate stress responses through a mechanism involving gamma-aminobutyric acid (GABA) and the vagus nerve (Bravo et al., 2011); and one where *Bacteroides fragilis* was found to have beneficial effects on behavior and gut barrier function in a mouse model of ASD (Hsiao et al., 2013).

Several mechanisms have been proposed for how gut microbiota could be interacting with the brain (Figure 1.1). The most direct of them is via spinal and vagal neurons; the vagal route in particular has been shown to be important in a number of studies (Bercik et al., 2011; Bravo et al., 2011; Dinan and Cryan, 2017; Forsythe et al., 2016). There have been various reports since the 1940s that bacteria can produce molecules analogous to mammalian neurotransmitters, for example acetylcholine (Stephenson and Rowatt, 1947), catecholamines (Asano et al., 2012), and GABA (Barrett et al., 2012), which could play a role in microbial gut–brain signalling. Another potentially important group of bacterial metabolites are short-chain fatty acids (SCFAs), especially propionate, butyrate and acetate, the main fermentative products of gut microbes (Koh et al., 2016). They can inhibit histone deacetylases and activate G protein-coupled receptors (Koh et al., 2016) and affect the enteric nervous system (Soret et al., 2010). It has even been suggested that acetate can cross the blood-brain barrier, with a central appetite-suppressing effect on the brain (Frost et al., 2014), although another study concluded that acetate produced by gut microbes has a hormonally mediated appetite-increasing effect instead (Perry et al., 2016); the discrepant results could be due to differences in model organisms and mode of acetate administration (Canfora and Blaak, 2017). Finally, the immune system is an important interface between gut microbes and the host when it comes to overall health, and could also mediate their influence on the CNS (El Aidy et al., 2014). However, although all these mechanisms seem plausible and have some experimental support, the evidence for their true importance remains limited (Forsythe et al., 2016; Hooks et al., 2018).

The boldest proponents for the importance of the microbiome–gut–brain axis are asking if our resident microbes might be actively manipulating our minds, even speculating on the potential economic impacts of such effects (Houdek, 2018). Others envision treating psychiatric illnesses with pre- or probiotics, called psychobiotics in this context (Dinan et al., 2013). There have been some small, preliminary intervention studies: for example, an open-label clinical trial with 18 ASD children found that microbiota transfer therapy – an approach based on filtered, standardized human stool preparations from healthy donors – improved behavioral symptoms (Kang et al., 2017), and a meta-analysis of randomized controlled trials concluded that probiotics could be beneficial in treating depression (Huang et al., 2016). More cautious views also exist, warning against premature extrapolation from rodent model experiments to humans (Hooks et al., 2018). Nevertheless, whether or not the strongest claims can be verified, there is a considerable body of evidence showing that some kind of a connection between gut microbes and the CNS does exist in mammals, offering a potential mechanism for linking gut microbiota to neurodegeneration.

## 1.2 Challenges of studying human microbiota

Although 16S rRNA gene sequencing based microbiota surveys have become popular enough to be called the current gold standard in the field, they have many potential stumbling blocks. Each step, from study design to interpreting the results, has its own set of practical details and choices to consider (Aho et al., 2015; Debelius et al., 2016; Goodrich et al., 2014; Knight et al., 2018; Pollock et al., 2018). These can make the results of different studies difficult to compare and has led to discussions of a "reproducibility crisis" (Schloss, 2018).

Most human microbiome studies are exploratory by nature. Performing power calculations beforehand to evaluate the required number of samples is challenging (Debelius et al., 2016); until recently (Kelly et al., 2015; Mattiello et al., 2016), there have been no specific tools for this. As it is, the simplest solution is to aim for as many samples as possible within practical

and financial limitations. Collecting information about potentially confounding clinical and lifestyle variables is also crucially important, so that these can be corrected for in the analyses (Debelius et al., 2016; Knight et al., 2018).

The choices of sample material and sampling technique were already touched upon in the previous section. Often, the most convenient sample type is chosen even though it might not truly represent the biologically active community; for example, stool for gut microbiota (Cani, 2018; Marchesi et al., 2016), and saliva for oral microbiota (Shi et al., 2018; Simón-Soro et al., 2013). A further question is how to store the samples after collection (Pollock et al., 2018). The effects of storage conditions have been studied particularly in the context of stool samples, supporting a common consensus of fast freezing and storage at -80 °C (Fouhy et al., 2015; Pollock et al., 2018). Several studies have shown that there are detectable differences in results depending on the choice of storage method, but typically, individual community variation is still easy to distinguish regardless of the storage effects (Bassis et al., 2017; Blekhman et al., 2016; Song et al., 2016).

The first step of the laboratory workflow is extracting DNA from the samples, which is typically done using commercial kits. Choosing the correct extraction approach for each sample material is crucial – for example, omitting a mechanical lysis step to break bacterial cell walls can have notable effects on the results (Brooks et al., 2015; Knudsen et al., 2016; Wagner Mackenzie et al., 2015; Walker et al., 2015), although the differences between kits can also be relatively minor (Rintala et al., 2017). Additionally, DNA extraction kits and other laboratory reagents may introduce contaminating bacterial DNA to the samples; these can be monitored by using technical control samples which contain no template DNA (Salter et al., 2014). Technical biases can also be estimated by sequencing mock microbial communities with a known composition (Bender et al., 2018; Bokulich et al., 2016; Brooks et al., 2015; Yeh et al., 2018).

The choice of PCR primers is another important consideration for amplicon-based studies. The length of the selected target region or regions of the 16S rRNA gene must correspond to that of the sequencing platform used (Klindworth et al., 2013). Different regions vary in how well they capture specific taxa; if there are particular taxonomic groups of interest, care should be taken to make sure that the selected primers detect them adequately (Castelino et al., 2017; Klindworth et al., 2013; Walker et al., 2015). The choice of the specific variable region targeted has been shown to have a much larger effect in the results of a gut microbiota analysis than the DNA extraction kit (Rintala et al., 2017).

The next step after PCR is DNA sequencing. Over the past decade, the most common sequencing platform for 16S rRNA gene surveys has moved from Roche's now defunct 454 pyrosequencing to Illumina MiSeq (D'Amore et al., 2016; Pollock et al., 2018). There are also other alternatives, such as Thermo Fisher's Ion Torrent and Ion Proton, and the Pacific Biosciences technology that can sequence full length 16S rRNA gene; so far, MiSeq seems to perform with the lowest error rate when compared to the other platforms (D'Amore et al., 2016). Similarly to the choice of PCR primers, the choice of sequencing platform has been shown to affect the end result of a study (Castelino et al., 2017; Clooney et al., 2016; D'Amore et al., 2016; Hahn et al., 2016). Additionally, even running every set of samples with the same sequencing machine will not eliminate all variation, as there can be run-specific differences (Bender et al., 2018; Yeh et al., 2018).

Each of the previously described steps – sample collection and storage, DNA extraction, PCR, and sequencing – has the potential to introduce a batch bias to the data. These can be compounded if, for example, samples collected at several separate points in time are handled in the laboratory in batches corresponding to these time points; this can, in turn, exacerbate the issue of reagent contamination if each batch is treated with a different kit lot (Salter et

al., 2014). Reassuringly, regardless of this long list of potential biases, several studies have shown that technical variation at specific steps is generally not so large that it would drown the true biological signal (Bassis et al., 2017; Bender et al., 2018; Blekhman et al., 2016; Song et al., 2016).

After sequencing, the data is in a raw format which needs to go through several computational steps, including trimming primers, removing low-quality sequences and chimeras, pairing reads if a paired-end sequencing approach is used, clustering into Operational Taxonomic Units (OTUs), and taxonomic classification (Kozich et al., 2013; Schloss et al., 2011; Schloss and Westcott, 2011). OTUs are a sequence similarity based proxy for species, traditionally built using a 97% sequence similarity cutoff (Knight et al., 2018). OTU clustering can be performed purely based on the sequences themselves (*de novo* approach), or in a reference database dependent manner (Goodrich et al., 2014). Recently, an alternative approach focusing on unique, exact sequence variants has begun to grow increasingly popular (Amir et al., 2017; Callahan et al., 2016; Knight et al., 2018). A variety of algorithms and reference databases are available for the taxonomic classification of sequences and OTUs. Most studies use general purpose reference databases, such as the Ribosomal Database Project (Cole et al., 2014). While there are stand-alone tools for each of the steps included in a 16S rRNA amplicon sequence analysis workflow, a more commonly used option are software packages that can perform all of them, typically either mothur (Schloss et al., 2009) or QIIME (Caporaso et al., 2010). The choices of sequence analysis tools, parameters, and reference databases can have drastic effects on the end result; for example, one study found that the amount of OTUs can vary by an order of magnitude depending on these choices (Koskinen et al., 2014).

After going through each of these steps, the data is ready for statistical analysis. It typically consists of a table of clinical data concerning the subjects, a table of taxonomic information for the OTUs, and a table of sequence read counts per subject and per OTU. The counts are usually normalized in some way to account for the differences in total amount of sequences in each sample. Several types of comparisons can then be performed to contrast various aspects of the microbial communities between groups of interest, focusing either on the whole community, often described with various diversity measures, or the abundances of specific taxa.

### 1.2.1 The characteristics of microbiota data

At the start of the statistical analysis part of a 16S rRNA gene amplicon sequencing study, the data describing the distributions of microbial OTUs is, at least on superficial inspection, count data: numbers of sequence reads representing each microbial taxon in each sample (Gloor et al., 2017; Tsilimigras and Fodor, 2016). Microbial ecology analyses build on traditional ecological research of similar data, consisting of counts of animals or plants within a specific sampling area (Gloor et al., 2017; St-Pierre et al., 2018). Count data presents challenges for statistical analyses, since it often does not meet the assumptions of popular statistical tests (St-Pierre et al., 2018). Typical ways to solve this issue in ecological studies are to either apply a transformation to make the data match the assumptions, or to use statistical approaches which can accommodate for the features of the data, particularly generalized linear models (GLM) and generalized linear mixed models (GLMM) (St-Pierre et al., 2018)

Both traditional (Martin et al., 2005) and microbial ecology (Paulson et al., 2013; Weiss et al., 2017) data often contain many zeros. This zero inflation can be either due to the true absence of a taxon from a sample, or technical reasons leading to the failure to detect the taxon in question (Martin et al., 2005). Another specific consideration

for microbiota analyses is that the number of total sequence reads produced per sample in amplicon sequencing studies, also known as the library size, can vary by orders of magnitude depending on many technical factors and even random chance (Gloor et al., 2017; McMurdie and Holmes, 2014; Weiss et al., 2017). These features of the data need to be accounted for in the downstream analyses to make sure that samples are truly comparable. Traditionally, microbiota studies have addressed the library size issue by using relative abundances (proportion of a taxon out of the total number of reads in a sample) or subsampling (randomly picking the same number of sequence reads from each sample; sometimes also called rarefying) (McMurdie and Holmes, 2014). Both of these approaches can diminish statistical power, leading some statisticians in the field to advocate for more sophisticated approaches, similarly to the use of GLMs in traditional ecology (McMurdie and Holmes, 2014).

Recently, several publications have brought up the issue that treating microbiota sequence data as counts analogous to those of any ecological study may not be the correct approach (Gloor et al., 2016; Gloor et al., 2017; Tsilimigras and Fodor, 2016). Since the library depth sets a limit to the total number of counts per sample, the number of counts for an individual taxon is not independent, and as the amplicon sequencing approach does not count the true numbers of microbial cells in the original samples, the data can only ever represent proportions of taxa (Gloor et al., 2017). Data of this type is called compositional, and microbial ecology tools that specifically consider the sequence read counts as such are becoming increasingly available (Gloor et al., 2016; Gloor et al., 2017; Tsilimigras and Fodor, 2016). The proponents of compositional data approaches have raised concerns that previous research may have reached incorrect conclusions, providing as an example a re-analysis of an autism-related mouse gut microbiota study (Hsiao et al., 2013), in which none of the reported results are statistically significant when compositional data analysis tools are used (Gloor et al., 2016).

### 1.2.2 Diversity analyses

A popular way of exploring microbial community patterns in environmental or human-related contexts is using various measures of diversity (Knight et al., 2018; Lozupone and Knight, 2008). These can be divided into comparisons of alpha and beta diversity, terms with a long history in traditional ecology, originally coined by R. H. Whittaker (Knight et al., 2018; Lozupone and Knight, 2008; Whittaker, 1972). Alpha diversity describes within-habitat diversity, and may include either only the species richness ("number of species in a sample of standard size") or both the richness and the evenness (the distribution of the detected species) (Whittaker, 1972). The original ecological definition of beta diversity is the change in species communities along environmental gradients; in other words, it is a measure of between-habitat diversity (Whittaker, 1972). Whittaker additionally defined a third type of diversity, gamma diversity, or the total diversity of a specific area, but this concept is typically not used in microbial ecology.

The traditional ecological concepts of diversity use counts of species (Whittaker, 1972). The targeted amplicon sequencing approach for microbes does not offer the resolution to accurately define species, so instead, analyses are usually done with the closest equivalent, OTUs (Lozupone and Knight, 2008). Commonly used alpha diversity measures in microbial ecology, inherited from traditional ecology research, include observed richness (the number of species or OTUs) and the Chao1 estimator (Chao, 1984), both of which only consider richness, and the Shannon (Shannon, 1948), Simpson (Simpson, 1949) and inverse Simpson indices, which include both richness and evenness (Goodrich et al., 2014; Knight et al.,

2018; Lozupone and Knight, 2008). An alternative to these measures are divergence-based methods which also take into account sequence similarity information, for example Faith's phylogenetic diversity (Faith, 1992). Alpha diversity metrics describe the diversity of a single sample, providing a sample-specific value. The distributions of the metrics between different groups of samples can then be compared with basic statistical tests.

Beta diversity metrics measure between-sample similarity, and as for alpha diversity, there is a large selection of them, most of them inherited from traditional ecology. Some, like the Jaccard similarity coefficient (Jaccard, 1912) and its complement, Jaccard dissimilarity, only take into account the presence or absence of taxa; others, such as Bray-Curtis dissimilarity (Bray and Curtis, 1957), also include abundance information (Barwell et al., 2015; Knight et al., 2018; Kuczynski et al., 2010). There are also measures specifically devised for microbial ecology, for example UniFrac (Lozupone and Knight, 2005), which considers phylogenetic information in addition to taxon counts. Since beta diversity metrics are calculated for pairs of samples, the resulting values form a distance matrix with values for each pair. Comparing these between groups requires specific statistical tests, for example permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001; Anderson, 2017). In addition to significance testing, the dissimilarity information can be used to cluster the samples or to visualize them using an ordination method, for example non-metric multidimensional scaling (NMDS) or principal coordinates analysis (PCoA) (Kuczynski et al., 2010). The various measures have been shown to vary in how well they capture the community patterns of microbial data (Kuczynski et al., 2010).

Diversity analyses can reveal overall community differences between the microbiota of different populations or in relation to specific variables, such as lifestyle factors and disease. However, a recent meta-analysis discovered that out of many disease-related alpha diversity differences reported in the literature, most did not hold when results were compared across studies (Duvallet et al., 2017). If the goal is to find differences between diseased and healthy states that could offer mechanistic explanations or lead to new therapeutic innovations, simply knowing that there is a difference in diversity is inadequate, and a more detailed understanding of the microbial community is required.

### 1.2.3 Differential abundance

Koch's postulates are an often – and possibly erroneously (Gradmann, 2014) – cited tenet of medical microbiology dating to the late 1800s. They state that to establish a causative link between a microbe and a disease, the microbe in question needs to be detected in an organism with the disease, isolated in pure culture, and used to reproduce the disease in another organism (Evans, 1976; Gradmann, 2014). Many scientists have later tried to fit these postulates into contexts other than their origin in bacterial infectious disease (Gradmann, 2014). In a sort of inversion of Koch's postulates, it has been suggested that to prove the beneficial status of a commensal microbe, it should be possible to isolate the specific strain and to show that it can ameliorate the condition of a diseased individual (Neville et al., 2018). Some human microbiome researchers have advocated for a whole-microbiome variant where it is the entire microbial community of an individual that can play the part of a causative agent (Vayssier-Taussat et al., 2014; Vonaesch et al., 2018; Zhao, 2013). Still, even when aiming for a community-wide view, understanding the community requires identifying the specific organisms and their roles in it (Neville et al., 2018; Vayssier-Taussat et al., 2014; White et al., 2009; Zhao, 2013).

One intuitively obvious way to determine which microbes are related to specific disease states is to observe how their numbers change between health and disease. Analogously to

differential expression of genes in RNA sequencing (RNA-seq) studies, the term differential abundance can be used to describe such a difference in the amount of microbes between groups of interest (McMurdie and Holmes, 2014). The question of how to best perform differential abundance comparisons is interlinked to the specific characteristics of 16S rRNA gene amplicon sequence data outlined earlier. It is possible, and quite commonplace, to compare the sequence counts with traditional statistical tests, such as the Welch two-sample t-test, the Wilcoxon rank-sum test, and the Kruskal-Wallis test (Hawinkel et al., 2017; Weiss et al., 2017). This is typically done on subsampled counts, leading to loss of a large quantity of data. An alternative approach recommended by some researchers (McMurdie and Holmes, 2014) is to utilize tools originally designed for RNA-seq studies, such as edgeR (Robinson et al., 2010) or DESeq2 (Love et al., 2014); they offer statistical tests where there is no separate normalization step prior to testing. Some tools have paid particular attention to the additional issue of zero-inflation in microbiota data, the most commonly used of them probably metagenomeSeq, which uses zero-inflated Gaussian (ZIG) mixture models (Paulson et al., 2013). More recently, the focus on the compositional nature of microbiota data (Gloor et al., 2017) has led to recommendations for using tools that take it into account, such as ALDEx2 (Fernandes et al., 2014) or ANCOM (Kaul et al., 2017; Mandal et al., 2015). Since there are typically thousands of OTUs or unique sequence variants in microbiota data sets, correcting for multiple comparisons is a common problem faced by all these tools (Hawinkel et al., 2017). Typically, it is addressed using one of a handful of $p$-value adjustment methods, such as the Benjamini and Hochberg (Benjamini and Hochberg, 1995) or Storey and Tibshirani (Storey and Tibshirani, 2003) false discovery rate (FDR).

Publications presenting new tools often offer analyses contrasting their performance to older ones, but such comparisons tend to be biased in favor of the new approach being described (Boulesteix et al., 2018). Three recent studies not related to any specific approach have attempted to benchmark the performances of differential abundance detection tools (Hawinkel et al., 2017; Thorsen et al., 2016; Weiss et al., 2017). Out of these, Thorsen et al. (2016) highlight that many tools result in high false positive rates, and that they do not seem to deal with the sparsity of the data adequately, concluding that edgeR and the feature model approach of metagenomeSeq are among the best-performing options. Hawinkel et al. (2017) agree on the overall unacceptably high false discovery rate of commonly used tools, also noting that the tools that control false discoveries better tend to have low power to detect differentially abundant taxa; their conclusion is not to recommend any specific test, but to advocate for more careful benchmarking when designing tools. Weiss et al. (2017) point out that the characteristics of the data used for benchmarking are important, since the tools' performance will vary depending on them, particularly mentioning DESeq2 and ANCOM as good approaches. To summarize these studies, there is currently no consensus for the best existing tool for detecting differentially abundant microbial taxa, and the best tool for a specific study may depend on its particular characteristics, such as the sample size, the study design, or the amount of inter-sample variability in the microbial communities.

## 1.3 Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease best known for its motor symptoms, such as slowness of movement (bradykinesia), rigidity, and rest tremor (Kalia and Lang, 2015). First described by James Parkinson in 1817 as "paralysis agitans or shaking palsy," the disease later came to carry his name based on the suggestion of another pioneering

researcher, Jean-Martin Charcot (Goetz, 2011). Although PD has been known for two hundred years and has been extensively researched, its cause remains a mystery. There are no good diagnostic tests for it, and no effective treatments to slow down its progression.

In the early years, several conditions with similar symptoms, nowadays distinguished as Parkinsonism-plus diagnoses, were grouped under the same name (Goetz, 2011). Since then, PD has been defined specifically as a disorder where the death of dopaminergic neurons in the substantia nigra pars compacta causes a dopamine deficiency, leading to the loss of dopamine in the basal ganglia, which results in the classic motor symptoms (Kalia and Lang, 2015). In addition to the destruction of dopaminergic neurons, another key feature of the disease is Lewy pathology (LP), the abnormal aggregation of the alpha-synuclein protein, which was first described a hundred years ago (Lewy, 1912; Goedert et al., 2012). It has been established that these protein aggregates contribute to the disease process; in fact, the first genetic mutation strongly associated with disease risk was in the alpha-synuclein gene (*SNCA*), discovered in an Italian family in 1997 (Polymeropoulos et al., 1997). The native state and physiological role of alpha-synuclein remain unclear, but it seems to be related to synaptic vesicle trafficking (Rocha et al., 2018). Similar protein aggregations may also be observed in neurologically healthy subjects, and there are many unanswered questions regarding the role of alpha-synuclein in the disease process, such as the exact mechanisms that make some forms of the protein toxic (Wong and Krainc, 2017). In addition to the key features of dopaminergic neuron death and LP, PD also involves changes in other brain regions, neurotransmitters other than dopamine, and protein aggregates other than LP (Kalia and Lang, 2015).

The presence of LP and neuron degradation in the substantia nigra pars compacta can be used for post-mortem confirmation of PD diagnosis (Kalia and Lang, 2015), but since these cannot be detected in a living patient, diagnosis is primarily based on expert identification of clinical features (Postuma et al., 2015). Differentiating between PD and atypical parkinsonisms, such as multiple system atrophy, progressive supranuclear palsy or corticobasal syndrome, is often challenging (Kalia and Lang, 2015; van Rumund et al., 2018). Modern neuroimaging techniques offer ways to exclude some of these conditions, but they tend to be expensive, are often difficult to interpret, and less useful at early disease stages, when changes are subtle (Kalia and Lang, 2015; Miller and O'Callaghan, 2015; van Rumund et al., 2018). Reliable and cost-effective biomarkers for PD are sorely needed (Miller and O'Callaghan, 2015).

Levodopa, the precursor molecule to dopamine, which was discovered in the 1960s (Goetz, 2011), is still the most important drug for managing the motor symptoms of PD. A number of other medications, such as dopamine agonists, monoamine oxidase type B inhibitors, and catechol-O-methyltransferase (COMT) inhibitors, are used in concert to alleviate the symptoms and to manage the complications of long-term dopaminergic treatment (Kalia and Lang, 2015). A promising newer approach is surgical treatment, particularly deep brain stimulation, which may improve quality of life more than medications in advanced stages of the disease (Kalia and Lang, 2015). So far, all these treatments are merely alleviating the symptoms of the disease; discovering a treatment that is neuroprotective or disease-modifying is a long-standing goal of the field.

### 1.3.1 Non-motor and premotor symptoms

In addition to its commonly known motor features, Parkinson's disease presents with a large variety of non-motor symptoms, such as anxiety, depression, fatigue, pain, cardiac autonomic dysfunction, cognitive impairment, hyposmia (decreased sense of smell), sexual dysfunction,

sleep disturbances, urinary urgency, and gastrointestinal issues, including sialorrhea (increased drooling), dysphagia (difficulty in swallowing), delayed gastric emptying, and constipation (Fasano et al., 2015; Goldman and Postuma, 2014; Schapira et al., 2017). Non-motor manifestations can dominate the clinical picture in some cases (Postuma et al., 2015). The symptoms appear at different stages of the disease process, following the decline of dopaminergic neurons, and some of them can manifest years or even decades before the motor onset that leads to diagnosis. In fact, the Movement Disorder Society has suggested a set of criteria describing a prodromal stage of PD based on these symptoms, although the criteria are meant for research, not for clinical use (Berg et al., 2015).

Among the most common prodromal symptoms of PD are mood disorders, REM sleep behavior disorder (RBD), olfactory dysfunction, and constipation (Goldman and Postuma, 2014). Anxiety and depression are common in PD patients (Schapira et al., 2017), affecting up to 43% (Dissanayaka et al., 2014; Pontone et al., 2009) and 35% (Reijnders et al., 2008) of subjects, respectively. A past history of either seems to be related to a higher disease risk (Goldman and Postuma, 2014). Regarding RBD, as many as 81% or 90% of subjects diagnosed with this sleep disorder go on to later develop a neurodegenerative disease, most commonly PD (Howell and Schenck, 2015; Iranzo et al., 2014; Schenck et al., 2013). Hyposmia affects over 90% of PD patients when compared to young subjects with a normal sense of smell (Doty, 2012; Haehner et al., 2009), and in one study, poor performance in olfactory testing led to a 5.2 odds ratio for PD (Webster et al., 2008); other studies have reported lower ratios, but the association is nevertheless striking (Goldman and Postuma, 2014). The prevalence estimates for constipation in PD patients vary widely, from as low as 8% to as high as 70%, in part due to methodological differences – objective measurements may result in higher detection rates than questionnaires (Knudsen et al., 2017). Since it commonly emerges long before diagnosis, constipation has also been suggested as a potential biomarker (Fasano et al., 2015; Stirpe et al., 2016).

### 1.3.2 Epidemiology and etiology

The prevalence and incidence of Parkinson's disease increase with older age, making age the single most important risk factor (Ascherio and Schwarzschild, 2016; Kalia and Lang, 2015; Wirdefeldt et al., 2011). Due to the aging global population, the disease is becoming more and more prevalent; one recent estimate suggests that in the United States alone, the population with PD will grow from the current approximate of 866 000 persons to almost two million by 2060 (Rodolfo et al., 2018). PD also seems to be slightly more common in males, and there are some ethnic and geographical differences (Ascherio and Schwarzschild, 2016; Kalia and Lang, 2015; Wirdefeldt et al., 2011).

In the search for the cause of PD, several genetic mutations related to disease risk have been described (Hernandez et al., 2016; Klein and Westenberger, 2012). They range from highly penetrant ones leading to a monogenic form of the disease, such as those in *SNCA*, to common ones with minor effects (Hernandez et al., 2016). A family history of PD was found to be the most important risk factor in a large meta-analysis, further underlining the significance of genetics (Noyce et al., 2012). However, as 90% of cases are thought to be sporadic, not familial (Klein and Westenberger, 2012), genetic factors are not enough to explain the entire picture of the disease. Instead, the etiology of the sporadic form of PD is thought to involve an interplay of genetic and environmental factors (Kalia and Lang, 2015; Wirdefeldt et al., 2011).

Numerous studies have explored the dietary and lifestyle factors and environmental exposures that could be related to the disease process. Among the best established risk

factors are past traumatic brain injury, history of melanoma, consumption of dairy products, and pesticide exposure (Ascherio and Schwarzschild, 2016; Noyce et al., 2012; Wirdefeldt et al., 2011). Some occupations, such as farming, teaching and healthcare professions, have also been linked to a higher risk, although the results from different studies are not consistent (Wirdefeldt et al., 2011). As for protective factors, tobacco smoking is one of the best established lifestyle factors that decrease disease risk (Ascherio and Schwarzschild, 2016; Noyce et al., 2012; Wirdefeldt et al., 2011). Several studies have also supported a protective role for consumption of coffee or alcohol (Ascherio and Schwarzschild, 2016; Bettiol et al., 2015; Noyce et al., 2012; Wirdefeldt et al., 2011). Disentangling these effects from confounding influences is challenging; for example, subjects with prodromal PD could have personality changes that make them less likely to smoke, or an altered response to nicotine (Wirdefeldt et al., 2011). Physical activity may lower the disease risk (Ascherio and Schwarzschild, 2016; Wirdefeldt et al., 2011). Most studies have found no connection between BMI and PD risk, but a recent Mendelian randomization study suggested that higher BMI might be protective (Noyce et al., 2017). There is also some evidence for a slightly lowered risk for individuals with high plasma cholesterol (Ascherio and Schwarzschild, 2016; Wirdefeldt et al., 2011). Some medications, including calcium channel blockers, statins, and nonsteroidal anti-inflammatory drugs, have been linked to lower risk of PD, while beta blockers are associated with a higher risk (Noyce et al., 2012). Finally, raised plasma urate might be protective against several neurodegenerative diseases, including PD (Paganoni and Schwarzschild, 2017). Since dairy products lower plasma urate (Choi et al., 2004), this has been suggested as a mechanism behind the increased risk conveyed by dairy consumption (Ascherio and Schwarzschild, 2016).

The main processes participating in the pathogenesis of PD are thought to include protein misfolding and disrupted protein handling (particularly related to alpha-synuclein), mitochondrial dysfunction, oxidative stress, impaired calcium handling, and neuroinflammation (Brundin and Melki, 2017; Rietdijk et al., 2017). There are hypotheses for how the epidemiological findings could be linked to the disease process, some of them supported by experimental evidence, such as an effect of nicotine on alpha-synuclein fibrillation, and caffeine, an adenosine receptor agonist, having neuroprotective properties (Wirdefeldt et al., 2011).

### 1.3.3 Suspicions of microbial involvement

In 2003, Braak and colleagues proposed in an influential article based on alpha-synuclein immunohistochemistry that the Lewy pathology of PD spreads through the brain in six stages (Braak et al., 2003a). They further suggested that the disease process begins in the olfactory bulb or the dorsal motor nucleus of the vagus nerve, initiated by an unknown neurotropic pathogen with prion-like properties (Braak et al., 2003b). Building on the early premotor symptoms of hyposmia and GI issues, they later expanded the hypothesis to specify that the possibly viral pathogen might invade the body using both nasal and gastrointestinal routes in a dual-hit manner (Hawkes et al., 2007). The GI invasion would occur via swallowed nasal secretions, which would lead to spreading of the pathology into the brain along the vagus nerve (Hawkes et al., 2007). This idea of externally initiated spread of pathology has become commonly known as Braak's hypothesis.

Since its publication, Braak's hypothesis has been discussed widely, and there is evidence both supporting and opposing it (Brundin and Melki, 2017; Hawkes et al., 2007; Lema Tomé et al., 2013; Rietdijk et al., 2017; Surmeier et al., 2017; Walsh and Selkoe, 2016). It has been demonstrated in animal models that alpha-synuclein pathology can spread from cell to

cell when fibrils are injected directly into the brain (Peelaerts et al., 2015; Shimozawa et al., 2017), intravenously (Peelaerts et al., 2015), intramuscularly (Sacino et al., 2014), or through the olfactory bulb (Rey et al., 2016). Even more intriguingly, experiments have shown that the pathology can spread from the GI tract to the brain in rats, with alpha-synuclein detected in the vagus nerve (Holmqvist et al., 2014), and in mice, with truncal vagotomy inhibiting the spread (Uemura et al., 2018). Epidemiological studies in humans showing a lowered risk of PD in subjects who have undergone truncal vagotomy offer further support (Liu et al., 2017; Svensson et al., 2015).

While the Braak staging pattern might accurately describe up to 83% of PD patients, it does not fit all of them, and there are individuals with Lewy pathology who never develop a neurodegenerative disease, as well as patients with PD who have no discernible LP (Berg et al., 2014; Braak and Del Tredici, 2017; Rietdijk et al., 2017; Surmeier et al., 2017; Walsh and Selkoe, 2016). Additionally, brain connectome analyses do not support the described pattern of spreading pathology (Surmeier et al., 2017). The main alternative to the pathogenic spread hypothesis focuses on cell-autonomous mechanisms, such as oxidative stress, and the specific vulnerability of affected neurons (Surmeier et al., 2017; Walsh and Selkoe, 2016). Of course, these two mechanisms need not be mutually exclusive; a synthesis would be that the spread of alpha-synuclein pathology follows a pattern along the network of at-risk neurons (Surmeier et al., 2017; Walsh and Selkoe, 2016).

Braak et al. originally proposed that the initial insult that sets off the alpha-synuclein aggregation is a pathogen, possibly a virus (Braak et al., 2003b; Hawkes et al., 2007). An alternative explanation is that this is a stochastic process without a particular causative agent (Brundin and Melki, 2017). Considering the epidemiological evidence pointing at both genetic and environmental risk factors, it might actually be the interplay of various different stressors leading to an increased likelihood of alpha-synuclein aggregation (Brundin and Melki, 2017). There could also be several subtypes of PD, with different mechanistic pathways leading to a similar outcome. Whichever the case, microbes could still be centrally involved in the processes underlying the disease, such as neuroinflammation and protein aggregation. In fact, exposure to curli amyloid protein-producing *Escherichia coli* has been shown to increase alpha-synuclein aggregation and neuroinflammation in rats (Chen et al., 2016b), prompting further research into a possible bacterial role in the disease process.

### 1.3.4 Microbial communities of interest

The Braak hypothesis and later studies discussing the potential associations of microbiota and PD, as well as the spectrum of premotor and non-motor symptoms of the disease, suggest a few microbial communities in the human body that are of particular interest. First of all, the common premotor symptom of hyposmia and the early involvement of the olfactory bulb, as well as the suggestion that a potential disease-causing agent could invade the body through the nose (Braak et al., 2003b; Doty, 2012; Haehner et al., 2009; Hawkes et al., 2007; Webster et al., 2008), make nasal microbiota a prime target.

Among the many non-motor symptoms of PD, patients often suffer from disturbances relevant to oral health, such as xerostomia (abnormal dryness of the mouth), sialorrhea and dysphagia (Barbe et al., 2016), and may have worse dental health than the general population, although the reports on this are somewhat conflicting (Rozas et al., 2017). Examining phosphorylated alpha-synuclein histopathology of post-mortem samples has revealed that the submandibular gland (Beach et al., 2010; Del Tredici et al., 2010) and the sensory terminals in the upper aerodigestive tract (Mu et al., 2015) are commonly affected by alpha-synuclein pathology. This has led to the suggestion that biopsies from

these locations, or even saliva, could offer methods for early detection of PD and other alpha-synucleinopathies (Adler et al., 2016; Beach et al., 2010; Del Tredici et al., 2010; Mu et al., 2015). One in vivo study of early PD cases (Adler et al., 2016) and another of RBD and PD patients (Vilas et al., 2016) have shown promise for submandibular needle biopsies. Considering the involvement of oral tissues in PD, the study of oral microbial communities could be relevant to understanding the disease.

The early gastrointestinal symptoms of PD (Fasano et al., 2015; Stirpe et al., 2016) could, on their own, present a reason to study gut microbiota in relation to the disease: whatever the cause of any potential microbial community disturbances, understanding them could offer new approaches for alleviating the GI symptoms. The possibility of a causal link between the gut and neurodegeneration presented in Braak's hypothesis (Hawkes et al., 2007), and the experimental evidence of vagotomy affecting PD risk (Holmqvist et al., 2014; Liu et al., 2017; Svensson et al., 2015; Uemura et al., 2018), give further grounds for targeting the microbes residing in the gut of PD patients. In addition to the motivation arising from the PD field, the recent interest in the microbiome–gut–brain axis provides further encouragement for exploring the involvement of gut microbes in neurodegenerative diseases in general, and PD in particular.

Out of other potentially interesting anatomical sites, one study has targeted bacterial DNA found in the blood of PD patients (Qian et al., 2018a), which could be a potential biomarker. Another location of interest for studying neurodegeneration and microbes could be the brain, and a few studies have reported detecting fungal and bacterial signatures in samples from Alzheimer's disease patients that differ from those of non-demented controls (Alonso et al., 2018; Emery et al., 2017). However, both of these sample materials are very challenging: the amount of microbial DNA in blood is very low, and brain studies can only be done using post-mortem tissue, which comes with a high risk of environmental microbial contamination. Due to the various challenges involved in all microbial amplicon sequencing studies, it is difficult to be certain whether the microbes extracted from such sample materials were truly present in the subjects' body, let alone what their clinical significance might be. An additional anatomical site of interest that represents a sampling challenge is the appendix; past appendectomy was recently shown to lower the risk of PD and delay the age of onset, potentially due to the alpha-synuclein aggregates and truncation products it contains (Killinger et al., 2018). The appendix has been suggested to act as a microbial reservoir for reseeding the colon (Girard-Madoux et al., 2018), and could therefore be involved in the potential link between gut microbiota, the brain, and PD.

# 2 Aims

The overarching aim of the Parkinson's disease microbiome project is to explore the potential association of PD and various bacterial communities inhabiting the human body. These bacteria could be either an agent influencing the development and progression or the disease, or a feature of the healthy body whose composition and functions are affected by the disease. A bacterium with an influence on the disease process could open venues towards developing new therapeutics, while one that shows a consistent difference between patients and control subjects, or patients in different subgroups (disease phenotypes, stable or progressed disease), could have applications for diagnosis or classification of patients.

Each of the studies included in this thesis explores microbiota at one of the anatomic locations that are of interest in relation to PD, with the following article-specific aims:

1. To compare gut microbiota of PD patients and control subjects to see if there are any differences in overall diversity or abundances of specific taxa (I).

2. To compare nasal and oral microbiota of PD patients and control subjects to see if there are any differences in overall diversity or abundances of specific taxa (II).

3. To explore the IBS-like gastrointestinal symptoms experienced by PD patients and their potential association to the abundances of gut microbial taxa (III).

4. To compare gut microbiota of 1) PD patients and control subjects and 2) progressed and stable PD patients at two time points in order to evaluate the temporal stability of their microbial communities, and the relations of overall diversity and abundances of specific taxa to disease progression (IV).

Determining which bacterial taxa differ between groups of interest (PD patients and control subjects, patients with or without IBS-like symptoms, or stable and progressed PD patients) is one of the main objectives of the project. The tools available for such comparisons make different assumptions regarding the data, have different statistical backgrounds, and can produce widely varying results. There is no gold standard for which one to use. These methods are the specific focus of my doctoral thesis, with the following aim:

5. To compare the performance of the differential abundance analysis approaches that we used for different parts of the PD microbiome project, ranging from simple case versus control comparisons to more complex attempts to correct for potential confounding variables while contrasting two time points.

# 3 Materials and methods

This chapter summarizes the key materials and methods of the four studies included in this thesis (Table 3.1), with a particular focus on the differential abundance detection tools. Further details are provided in each article. Article III was based on the sequence data produced for article I, and the descriptions of the laboratory and sequence analysis steps for I also apply for III.

## 3.1 Study subjects, clinical data and sample collection

At the start of the project, between November 2011 and December 2012, we recruited a total of 277 subjects from the in- and outpatient departments of Helsinki University Central Hospital and Hyvinkää Hospital, via referrals from cooperating neurologists and other participants, and invitations in patient journals and meetings. Patients' PD diagnosis was defined based on the Queen Square Brain Bank criteria. Control subjects were sex- and age-matched to PD patients, and were not allowed to have any signs of parkinsonism or potential premotor symptoms. Other exclusion criteria for both groups included having any first-degree relatives with PD, active smoking within the past 6 months, and antibiotic use within the past month. 76 PD patients and 76 control subjects passed the selection criteria and were chosen for the project. We used samples from these subjects in some or all of the studies, with the total number of subjects per analysis ranging from 148 (II, oral microbiota analysis) to 128 (IV, follow-up gut analysis) (Table 3.1). Samples from specific subjects were excluded from particular studies for various clinical and technical reasons, such as surgery prior to follow-up sampling, or samples failing to produce enough PCR amplicons or sequence reads.

In addition to providing samples for microbiota characterization and basic information on their medications and medical conditions, the study subjects took several questionnaires targeting specific aspects of their health which could be related to microbiota, PD symptoms, or both. These included the Wexner constipation score (Agachan et al., 1996), the Rome III questionnaire for functional bowel disorders (Longstreth et al., 2006), the Swallowing Disturbance Questionnaire (SDQ; Lam et al., 2007), the Sialorrhea Clinical Scale for PD (SCS-PD; Perez Lloret et al., 2007), the REM sleep behavior disorder screening questionnaire (RBDSQ; Stiasny-Kolster et al., 2007), the Non-Motor Symptoms Scale (NMSS; Chaudhuri et al., 2007), and the Sniffin' Sticks 16-item smell identification score (Boesveldt et al., 2009)). At follow-up, the subjects also took a 163 item Food Frequency Questionnaire (FFQ; based on Pietinen et al., 1988). PD patients' parkinsonian symptoms were assessed with the Unified Parkinson's Disease Rating Scale (UPDRS; Fahn et al., 1987), which includes both clinicians' and subjects' evaluations, and their medication load was calculated as Levodopa Equivalent Dose (LED; Tomlinson et al., 2010). In the follow-up gut study (IV), we classified the PD patients as stable or progressed based on how their UPDRS I-III sum score and LED score had changed between the time points.

Sampling for the pilot study took place between December 2011 and April 2013, and follow-up sampling on average 2.25 years later. At both time points, subjects collected stool

Table 3.1: Summary of methods used in this thesis.

| | Article I | Article II | Article III | Article IV |
|---|---|---|---|---|
| **n (PD patients / control subjects)** | 72 / 72 | oral: 72 / 76, nasal: 69 / 67 | 72 / 0 | 64 / 64 |
| **Time points** | 1 | 1 | 1 | 2 |
| **Main contrasts explored with microbial data** | PD / control | PD / control | | PD / control |
| | PD only: disease phenotype | | PD only: IBS +/- | PD only: progressed / stable |
| **Sample material** | Stool | Oral and nasal swabs | | Stool |
| **DNA extraction kit** | PSP Spin Stool DNA Plus Kit | FastDNA Spin Kit for Soil | | PSP Spin Stool DNA Plus Kit |
| **16S rRNA gene target region** | V1-V3 | V1-V3 | | V3-V4 |
| **Forward PCR primer(s)** | pA (Lane 1991): **AGAGTTTGATCMTGGCTCAG** | pA (Lane 1991, modified): **AGAGTTTGATCMTGGCTCAG**, **TAGAGAGTTTGATCMTGGCTCAG**, **CTCTAGAGTTTGATCMTGGCTCAG** | | 341F (Klindworth et al. 2013, modified): **CCTACGGGNGGCWGCAG**, **GTCCTACGGGNGGCWGCAG**, **AGAGCCTACGGGNGGCWGCAG**, **TAGTGTCCTACGGGNGGCWGCAG** |
| **Reverse PCR primer(s)** | pD' (Edwards et al. 1989): **GTATTACCGC-GGCTGCTG** | pD' (Edwards et al. 1989, modified): **GTATTACCGCGGCTGCTG**, **CGTATTACCGCGGCTGCTG**, **TAGTATTACCGCGGCTGCTG** | As in Article I (the same data was used) | 785R (Klindworth et al. 2013, modified): **GACTACHVGGGTATCTAATCC**, **AGACTACHVGGGTATCTAATCC**, **TCTGACTACHVGGGTATCTAATCC**, **CTGAGTGGACTACHVGGGTATCTAATCC** |
| **Sequencing platform** | 454 (single-end) | MiSeq (paired-end) | | MiSeq (paired-end) |
| **Untrimmed sequence reads** | 2 549 217 | oral: 21 645 150, nasal: 8 638 162 | | 34 701 899 |
| **Trimmed sequence reads** | 1 131 504 | oral: 1 997 846, nasal: 3 611 736 | | 18 867 278 |
| **European Nucleotide Archive accession number** | PRJEB4927 | PRJEB14536 | | PRJEB27564 |
| **Subsampling (sequence reads per sample)** | For all analyses (4 500) | Only for beta diversity analyses (oral: 5 736, nasal: 2 197) | None | Only for beta diversity analyses (2 201) |
| **Alpha diversity** | Chao1, ACE, Shannon, inverse Simpson | Shannon and inverse Simpson | Not compared | Observed richness, Shannon and inverse Simpson |
| **Beta diversity** | Yue & Clayton theta, Morisita-Horn and Bray-Curtis with Unifrac | Bray-Curtis dissimilarity with adonis | Not compared | Bray-Curtis dissimilarity with adonis and envfit |
| **Differential abundance tools** | Metastats | DESeq2 | DESeq2 | DESeq2, ANCOM, random forests |
| **Taxonomic levels for differential abundance** | Family | OTU, genus and family | OTU, genus and family | OTU, genus and family |

samples (I, IV) at home into collection tubes pre-filled with Stool DNA Stabilizer (PSP Spin Stool DNA Plus Kit, STRATEC Molecular). Subjects were instructed to store the samples in the refrigerator until delivery to the clinic (a maximum of 3 days). Once received, we placed the samples in -80 °C until further processing. The baseline samples were thawed twice, first for the initial DNA extraction for the pilot study (I) and then a second time for the follow-up study (IV). Nasal and oral swab samples (II) were collected by medical professionals during clinic visits. The tips of the cotton sampling swabs were deposited in small aliquot containers and on ice immediately, and transferred to -80 °C within 20 minutes.

## 3.2 DNA extraction, PCR and sequencing

For the gut microbiota analyses (I, IV), we extracted total DNA from stool samples using the PSP Spin Stool DNA Plus Kit (STRATEC Molecular). For the oral and nasal swab samples (II), the FastDNA Spin Kit for Soil (MP Biomedicals) was used instead.

In all studies, we used a two-step PCR approach where the first round was run with universal bacterial primers targeting specific variable regions of the 16S rRNA gene (Table 3.1): V1-V3 for I and II (Edwards et al., 1989; Lane, 1991) and V3-V4 for IV (Klindworth et al., 2013). The Illumina workflow versions of the universal 16S rRNA gene primers had additional nucleotides for better mixing in sequencing. The first PCR round also included either 18-mer overhangs (I) or partial Illumina TruSeq adapter sequences (II, IV). The second round of PCR used primers targeting the 18-mer overhangs with added pyrosequencing barcodes (I), or full-length Illumina adapters and indices (II, IV). The PCR protocol was the same in all projects, consisting of initial denaturation at 98 °C for 30 s, 15 cycles at 98 °C for 10 s, 65 °C for 30 s, 72 °C for 10 s, and a final extension for 5 minutes for the first round. Similar conditions but with 18 cycles were used for the second round.

We sequenced the baseline stool sample amplicons (I) using a single-end pyrosequencing approach with the 454-GS FLX Titanium chemistry (Roche Diagnostics). For the other studies, we performed paired-end sequencing on the Illumina MiSeq platform (II: v2 600 cycle kit, IV: v3 600 cycle kit), with 325 base pairs for the forward and 285 base pairs for the reverse read. To make the baseline samples comparable to the follow-up samples, we resequenced them on the MiSeq platform simultaneously with the follow-up samples (IV).

## 3.3 Bioinformatic sequence analysis

For all MiSeq sequence data, we removed adapters and primers with cutadapt (Martin, 2011). In the oral and nasal sequence data analysis, we also used it to perform additional quality trimming (II). For the follow-up gut sequence data (IV), parameter testing suggested that additional trimming did not in fact improve the end result considerably, and we omitted the added quality control parameters.

mothur (Schloss et al., 2009) was our program of choice for the 16S rRNA gene amplicon sequence analysis for all studies, including denoising (I), pairing paired-end sequences (II, IV), trimming bad quality reads and chimeras, taxonomical classification, and clustering sequences into Operational Taxonomic Units (OTUs). Each analysis followed the corresponding Standard Operating Procedure (SOP) for mothur: the 454 version (mothur: Standard Operating Procedure for 454 sequenced 16S data, 2014) for the baseline gut analysis (I), and the MiSeq version (Kozich et al., 2013; mothur: Standard Operating Procedure for MiSeq sequenced 16S data, 2017) for the nasal, oral, and follow-up gut analyses (II, IV), with some adjustments to parameters according to the specific

characteristics of each data set. Details on sequence analysis workflows, including the versions of software and reference databases, can be found in each article.

Even though the mothur workflow for the baseline gut article (I) included OTU clustering, we decided to focus our analyses on family-level data (the approach described as "phylotypes" in the SOP), and to subsample the data to 4 500 sequences per sample. However, we used non-subsampled OTU-level data in additional analyses, including the comparisons of IBS-like symptoms in PD patients (III), and previously unpublished differential abundance detection tool comparisons (subsection 4.1.2 of this thesis). The MiSeq sequence data sets (II, IV) were not binned to phylotypes or subsampled at this stage.

## 3.4 Statistics

The majority of the statistical analyses in the baseline gut article (I) were performed using either mothur or IBM SPSS Statistics. For the other three studies, we used the R statistical programming language (R Core Team, 2018) for all visualizations and statistical tests, with the R package phyloseq (McMurdie and Holmes, 2013) as the main tool for microbiota data management. Information regarding the specific versions of R and the packages used in each study can be found in the articles. Additional analyses that were used in specific studies, such as oligotyping (II) and enterotype classification (IV), are not introduced here, as they are not discussed in this thesis.

### 3.4.1 Diversity analyses

#### Alpha diversity

We calculated alpha diversity indices with mothur for the baseline gut study (I) and with the phyloseq R package for the other two studies that included diversity comparisons (II, IV). We compared the Shannon and inverse Simpson indices, which take into account both richness and evenness, in each of the three studies. Additionally, we also tested the richness estimators Chao1 and ACE at baseline (I), and observed richness in the follow-up gut study (IV). We used the Kruskal-Wallis (Kruskal and Wallis, 1952) and Wilcoxon rank-sum (Mann and Whitney, 1947; Wilcoxon, 1945) tests to statistically compare alpha diversities between groups of interest.

#### Beta diversity

Similarly to the alpha diversity analyses, we performed the baseline gut (I) beta diversity comparisons with mothur, and the comparisons for the other studies (II, IV) with the R packages phyloseq and vegan (Oksanen et al., 2015). We used subsampled sequence data for all beta diversity analyses (the general 4 500 sequence cutoff for I, and the lowest number of sequence reads in a sample for II and IV; Table 3.1). The approach used in the baseline study was overall different from the others, and included comparing Unifrac distances of the Yue & Clayton theta, Morisita-Horn and Bray-Curtis dissimilarities (similarly to the mothur SOP; mothur: Standard Operating Procedure for 454 sequenced 16S data, 2014). In both other studies where beta diversity was explored, comparisons were based on Bray-Curtis dissimilarity, and PERMANOVA (the "adonis" command from vegan) was used for statistical significance testing. For the oral and nasal data (II), beta diversity was only calculated on the OTU level; for the follow-up gut data (IV), we also calculated it on the genus and family levels, and included an additional statistical test ("envfit," also from vegan).

Table 3.2: Differential abundance detection tools used in this thesis.

| | Publication(s) | Normalization | Statistical background | Multiple comparison correction | Confounder correction available | Used in article |
|---|---|---|---|---|---|---|
| **Metastats** | White et al. 2009 | Relative abundance | Nonparametric t-test (common features), Fisher's exact test (sparse features) | S-T | No | I |
| **LEfSe** | Segata et al. 2011 | Relative abundance times 1000 | K-W (groups), Wilcoxon rank-sum test (subgroups), LDA (effect size) | None | No | Unpublished |
| **metagenomeSeq** | Paulson et al. 2013 | Cumulative-sum scaling | ZIG mixture model | B-H | Yes | Unpublished |
| **DESeq2** | Love et al. 2014 | Size factors in models | Negative binomial GLMs | B-H | Yes | II, III, IV |
| **ANCOM** | Mandal et al. 2015, Kaul et al. 2017 | Log transformation | K-W or Friedman's test (depending on design) | B-H | Yes (some versions) | IV |
| **Random forests** | Breiman 2001, implemented with Liaw & Wiener 2002, Murphy et al. 2010, Archer 2016 | Not applicable | Supervised machine learning using ensembles of decision trees | Not applicable | Not applicable | IV |

K-W: Kruskal-Wallis test; LDA: linear discriminant analysis; ZIG: zero-inflated Gaussian;
GLM: generalized linear model; S-T: Storey and Tibshirani false discovery rate;
B-H: Benjamini and Hochberg false discovery rate

## 3.4.2 Differential abundance

Over the course of the Parkinson's disease microbiome project, we have used several tools to look for differentially abundant taxa (Table 3.1). Each has its own statistical background (Table 3.2), its advantages, and disadvantages. Many of these tools come with built-in normalization procedures and multiple comparison correction. This subsection offers a brief introduction to these tools, including two that were only used in previously unpublished comparisons presented in this thesis.

### Metastats

Metastats (White et al., 2009) is a differential abundance comparison method designed for clinical microbiota studies. Metastats always compares two groups, and therefore is not applicable to numeric variables of interest. Its default normalization approach is to convert data into relative abundances. For differential abundance testing, Metastats uses a nonparametric t-test approach: after calculating the t-statistic for the actual data, the target variable labels of the taxon abundance matrix are randomly permuted to re-calculate the t-statistic multiple times, and the result is considered the null distribution for estimating the $p$-value (Storey and Tibshirani, 2003). The $p$-values are multiple comparison corrected with the Storey and Tibshirani FDR (Storey and Tibshirani, 2003). For low-abundance taxa, defined as those where the total number of observations in a group is less than the total number of subjects in that group, Metastats uses Fisher's exact test instead.

Metastats was the main differential abundance comparison tool used in our baseline gut

study (I). The contrasts tested in the article using family level data included PD patients to control subjects, PD patients with the tremor dominant phenotype to those with the postural instability and gait difficulty phenotype, and subjects with a history of transient ischemic attack (TIA) / ischemic stroke or atrial fibrillation to those who didn't have these conditions. All analyses were performed via the mothur wrapper of Metastats, except for the TIA / ischemic stroke and atrial fibrillation comparisons, which used the now defunct online platform. The default number of permutations for the t-statistic calculation is 1 000, and we did not change this default value in our comparisons. Since Metastats can only be used to compare two groups and does not offer ways to correct for confounding variables, we additionally used generalized linear models (GLMs), implemented in IBM SPSS Statistics, to explore the effects of potential confounders on the taxa detected by Metastats. In addition to the published results, we also used Metastats for OTU level comparisons of PD patients to control subjects which have not been published previously.

### DESeq2

DESeq2 (Love et al., 2014) is an R package originally designed for estimating dispersions and fold changes in RNA-seq studies. Since 16S rRNA gene amplicon sequence data presents some of the same challenges as RNA-seq data, such as the need to correct for different library sizes, DESeq2 has been adopted by microbiota researchers, and has been shown to perform as well as some of the tools specifically written with amplicon studies in mind (McMurdie and Holmes, 2014). The statistical approach of DESeq2 is to fit GLMs with the negative binomial probability distribution and the logarithmic link function. This is done separately for each feature (genes in RNA-seq studies, microbial taxa in microbiota comparisons). The input count data are used for modeling in a non-normalized form, but models include normalization constants, or size factors, calculated for each sample to correct for the different library sizes. DESeq2 also estimates a feature-specific dispersion parameter that is included in the models to correct for within-group variability. Additionally, it applies a logarithmic fold change (LFC) estimate shrinkage step to account for the strong variance of features with low read counts. The shrunken LFC values and their standard errors are used for the final Wald test for differential abundance. The default output tables include $p$-values adjusted for multiple comparisons with the Benjamini and Hochberg FDR approach (Benjamini and Hochberg, 1995).

DESeq2 was the only differential abundance comparison tool we used in the oral and nasal microbiota comparisons (II), and the comparisons of gut data for IBS-like symptoms in PD patients (III). We also used it as one out of several tools in the unpublished baseline gut comparisons, and the follow-up gut analyses (IV). As DESeq2 lets the user specify any statistical model they choose, each set of comparisons was performed with a different model (Table 3.3), ranging from simple two-group contrasts (unpublished results and III) to models with up to seven confounders (II) and with interactions (IV). We always ran DESeq2 comparisons on three taxonomic levels (OTU, genus and family), except for the unpublished results, where the genus level was omitted.

The comparisons of Parkinson's patients to control subjects for the follow-up study (IV) were a special case: they included two time points from the same subjects, and we wanted to include subject identity in the model. We also wanted to use BMI as a confounder, but some subjects lacked values for this variable at baseline, leaving an uneven number of PD patients and control subjects. DESeq2 does not accept missing values, and the specific model we had chosen (Table 3.3) required matching numbers of subjects in the control and PD groups. Therefore we ran the comparisons with a leave-one-out approach, excluding each of the 62

Table 3.3: Models used in the DESeq2 comparisons.

| Article | Subset | Models |
|---|---|---|
| unpublished | all | taxon ~ **PD status** |
| II | all (oral) | taxon ~ DNA extraction date + sex + age + history of ENT surgery + alcohol units per week + SDQ total + SCS-PD total + **PD status** |
| | all (nasal) | taxon ~ DNA extraction batch + sex + age + history of ENT surgery + Sniffin' Sticks score + **PD status** |
| III | PD only | taxon ~ **IBS**<br>taxon ~hypothyroidism + lactose intolerance + sex + BMI + dopamine agonist use + tremor score Jankovic + **IBS** |
| IV | all | taxon ~ Rome III score + BMI +<br>**PD status** : subject + timepoint * **PD status** |
| | PD only | taxon ~ COMT + **progression**<br>(model run separately for baseline and follow-up) |

Main contrast of interest in bold font.
PD status: Parkinson's patient/control subject; ENT surgery: non-major ear, nose or throat surgery; SDQ: swallowing disturbance questionnaire; SCS-PD: sialorrhea clinical scale for Parkinson's disease; IBS: Rome III score based IBS criteria fulfilled (yes/no); BMI: body mass index; COMT: cathecolamine-O-methyl transferase inhibitor medication; progression: PD progression defined by changes in symptoms and medication (progressed/stable)

PD patients in turn. The average FDR-adjusted $p$-value of these 62 rounds was considered as the final result.

### LEfSe

LEfSe (linear discriminant analysis (LDA) effect size; Segata et al., 2011) is a differential abundance comparison algorithm available through the Galaxy platform (Afgan et al., 2016). It was specifically designed for biomarker discovery in metagenomic data. LEfSe can be used to compare factor variables with multiple levels and with subclasses, but not numeric variables, and it does not offer confounder correction. LEfSe uses the Kruskal-Wallis rank-sum test to detect differentially abundant features between all groups, followed by the unpaired Wilcoxon rank-sum test for significance testing between specific subgroups. Finally, it uses LDA to estimate effect sizes for the detected features. It does not include multiple comparison correction.

We tested LEfSe in the previously unpublished analysis of baseline gut data, comparing PD patients and controls on the family and OTU levels. The comparisons were run on non-subsampled data, implementing the normalization procedure offered by the online platform: converting the data to relative abundances and multiplying by 1000.

### metagenomeSeq

The R-based metagenomeSeq (Paulson et al., 2013) is designed for analysis of sparse data, such as 16S rRNA gene amplicon sequence count tables, and provides a normalization method and a statistical testing approach. The normalization, cumulative-sum scaling, is based on dividing the raw counts by the cumulative sum of counts up to a specific percentile, which is selected based on the data at hand. The statistical testing approach is ZIG mixture modeling. Its aim is to account for the large number of zeros present in this type of data, which is often caused by undersampling instead of actual absence of taxa. The ZIG models are fit

separately for each taxon, and since the models can include as many variables as desired, confounders can be included in analyses. The default multiple comparison correction method offered by metagenomeSeq is the Benjamini and Hochberg FDR.

We tested metagenomeSeq in the previously unpublished analysis of the baseline gut data. Specifically, we contrasted PD patients to control subjects on the family and OTU levels using the fitZig command, with a maximum of 25 iterations for the expectation-maximization algorithm used to evaluate the model fits. We trimmed the output data to only include taxa that have at least the average number of effective samples compared to the full data.

### ANCOM

ANCOM (analysis of composition of microbiomes; Mandal et al., 2015) is, as its name states, a tool whose approach is to treat microbiota data as compositional. Before testing, ANCOM log-transforms the count data after first adding 1 to all taxa, to manage those with a count of 0. The differential abundance detection uses basic statistical tests, including the Kruskal-Wallis rank-sum test, ANOVA, and Friedman's test for repeated measures. ANCOM is available as an R script, and its output is a list of taxa detected as differentially abundant, without $p$-values. It offers two multiple comparison correction options, a more conservative correction for the full table, and a less stringent taxon specific approach. As of ANCOM-II (Kaul et al., 2017), there are also options for trimming data before analysis to exclude those taxa that have a high proportion of zeros.

We performed our analyses using an updated but unpublished version of ANCOM (ANCOM 2.0: updated R code, 2018), since the ANCOM-II article had not yet been published at the time when we began working with the data, and the unpublished version includes features not available in the published versions, most importantly confounder correction. ANCOM was only used in the follow-up gut analyses (IV), where we used it to contrast PD patients to controls and progressed patients to stable patients on the OTU, genus and family levels. As recommended by the authors, we chose the second, less stringent option for multiple comparison correction, and used 0.9 as the cutoff for trimming taxa with zeros.

### Random forests

Unlike all other approaches introduced in this section, random forests (Breiman, 2001) is not a differential abundance detection tool, but a supervised learning algorithm that can be used for countless different tasks. There are no microbiota specific tools that use the algorithm, but it can be implemented in various programming languages. For example, there are multiple R packages that offer tools for random forests analyses. Random forests have been shown to perform well in microbiota classification (Knights et al., 2011; Statnikov et al., 2013), and have been used in many published microbiota studies (for example, Duvallet et al., 2017; Lehtimäki et al., 2017; Sze and Schloss, 2018).

We used random forests for classifying our data to the groups of interest (PD patients or control subjects, progressed or stable PD patients) based on OTU, genus and family level microbiota data in the follow-up gut study (IV). The classifiers were built with the randomForest R package (Liaw and Wiener, 2002). To assess the significances of specific taxa, we used rfPermute (rfPermute 2.1.5, 2016). Additionally, we evaluated the the success of the classifiers at correctly placing the subjects into the groups of interest with rfUtilities (Murphy et al., 2010).

# 4 Results

## 4.1 Baseline gut study: Case versus control

### 4.1.1 First family-level results (I)

The primary goal of our baseline gut study was to determine whether there are differences in overall community structure or specific microbial taxa in the gut microbiota of PD patients compared to control subjects. For the published analyses, we focused on family level data; this choice was made mainly to simplify the reporting of results.

We found no differences in alpha diversity between patients and control subjects for any of the indices we tested (Chao1, ACE, Shannon, and inverse Simpson). In beta diversity comparisons of three indices (Yue & Clayton measure of dissimilarity, Morisita–Horn similarity coefficient, and Bray–Curtis dissimilarity), the PD and control groups differed significantly with each index ($p < 0.02$ for all comparisons).

Differential abundance comparisons contrasting PD patients to control subjects with Metastats gave a list of six families with an unadjusted $p$-value $< 0.05$, but only

Table 4.1: Differential abundance results of article I. A. Metastats and the relative abundances of the detected taxa; B. Generalized linear models with confounding variables for families that had a significant uncorrected $p$-value with Metastats.

**A.**

| Family | Relative abundance (mean ± SD %) PD | Controls | Metastats $p$-value (PD/control) Unadjusted | Adjusted |
|---|---|---|---|---|
| *Prevotellaceae* | 2.70 ± 0.32 | 12.06 ± 3.73 | 0.001 | 0.031 |
| *Lactobacillaceae* | 0.44 ± 0.04 | 0.02 ± 0.00 | 0.004 | 0.063 |
| *Verrucomicrobiaceae* | 0.06 ± 0.00 | 0.02 ± 0.00 | 0.014 | 0.146 |
| *Bradyrhizobiaceae* | 0.16 ± 0.00 | 0.03 ± 0.00 | 0.021 | 0.151 |
| *Clostridiales incertae sedis* IV | 2.49 ± 0.30 | 1.01 ± 0.03 | 0.025 | 0.151 |
| *Ruminococcaceae* | 33.63 ± 1.99 | 28.54 ± 1.81 | 0.029 | 0.151 |

**B.**

| Family | GLM $p$-value PD status | AFib | TIA | Warfarin | Statins | NMSS | Wexner | *Prevotellaceae* abundance | COMT |
|---|---|---|---|---|---|---|---|---|---|
| *Prevotellaceae* | <0.001 | 0.358 | 0.927 | 0.791 | 0.671 | 0.696 | 0.736 | | |
| *Lactobacillaceae* | <0.001 | 0.983 | <0.001 | 0.764 | <0.001 | 0.320 | 0.911 | 0.002 | <0.001 |
| *Verrucomicrobiaceae* | 0.001 | 0.247 | 0.525 | 0.066 | 0.783 | 0.020 | 0.003 | 0.001 | |
| *Bradyrhizobiaceae* | <0.001 | 0.858 | 0.279 | 0.768 | 0.049 | 0.024 | <0.001 | 0.094 | |
| *Clostridiales incertae sedis* IV | 0.001 | 0.565 | 0.737 | 0.171 | <0.001 | 0.266 | 0.187 | <0.001 | <0.001 |
| *Ruminococcaceae* | 0.697 | 0.315 | 0.485 | 0.487 | 0.331 | 0.677 | 0.093 | <0.001 | |

PD status: Parkinson's patient/control subject; AFib: Atrial fibrillation; TIA: transient ischemic attack or ischemic stroke; NMSS: total non-motor symptoms scale score; Wexner: total Wexner constipation score; COMT: cathecolamine-O-methyl transferase inhibitor medication

*Prevotellaceae* had a multiple comparisons adjusted *p*-value below the cutoff (Table 4.1A; I: Supplementary eTable 4). *Prevotellaceae* was also the only family out of six which was more abundant in control subjects (Figure 4.1A); the other five were more abundant in PD patients.

The six families with a significant unadjusted *p*-value were further compared with GLMs to explore potential confounding variables: history of atrial fibrillation, history of TIA or stroke, warfarin medication, statin medication, NMSS score, Wexner constipation score, and COMT inhibitor medication. Taxa other than *Prevotellaceae* were corrected for *Prevotellaceae* abundance to evaluate whether the change in abundance might be a compensatory effect related to decreased *Prevotellaceae*. None of the confounders were significant in the model for *Prevotellaceae*, suggesting that the main variable affecting the abundance of this family was PD status (Table 4.1B; I: Table 2). Out of the other families, all but *Ruminococcaceae* were significantly associated with PD status, although they were also influenced by several confounders. The abundance of *Ruminococcaceae* seemed to depend mainly on the abundance of *Prevotellaceae*.

### 4.1.2 Additional differential abundance comparisons

Since the first differential abundance comparisons were only performed with Metastats on the family level, we wanted to repeat these comparisons on the OTU level and with other tools. To do this, we compared OTU and family level baseline gut data using three additional tools: LEfSe, metagenomeSeq, and DESeq2. We also ran Metastats on the OTU level. These results have not been published previously.

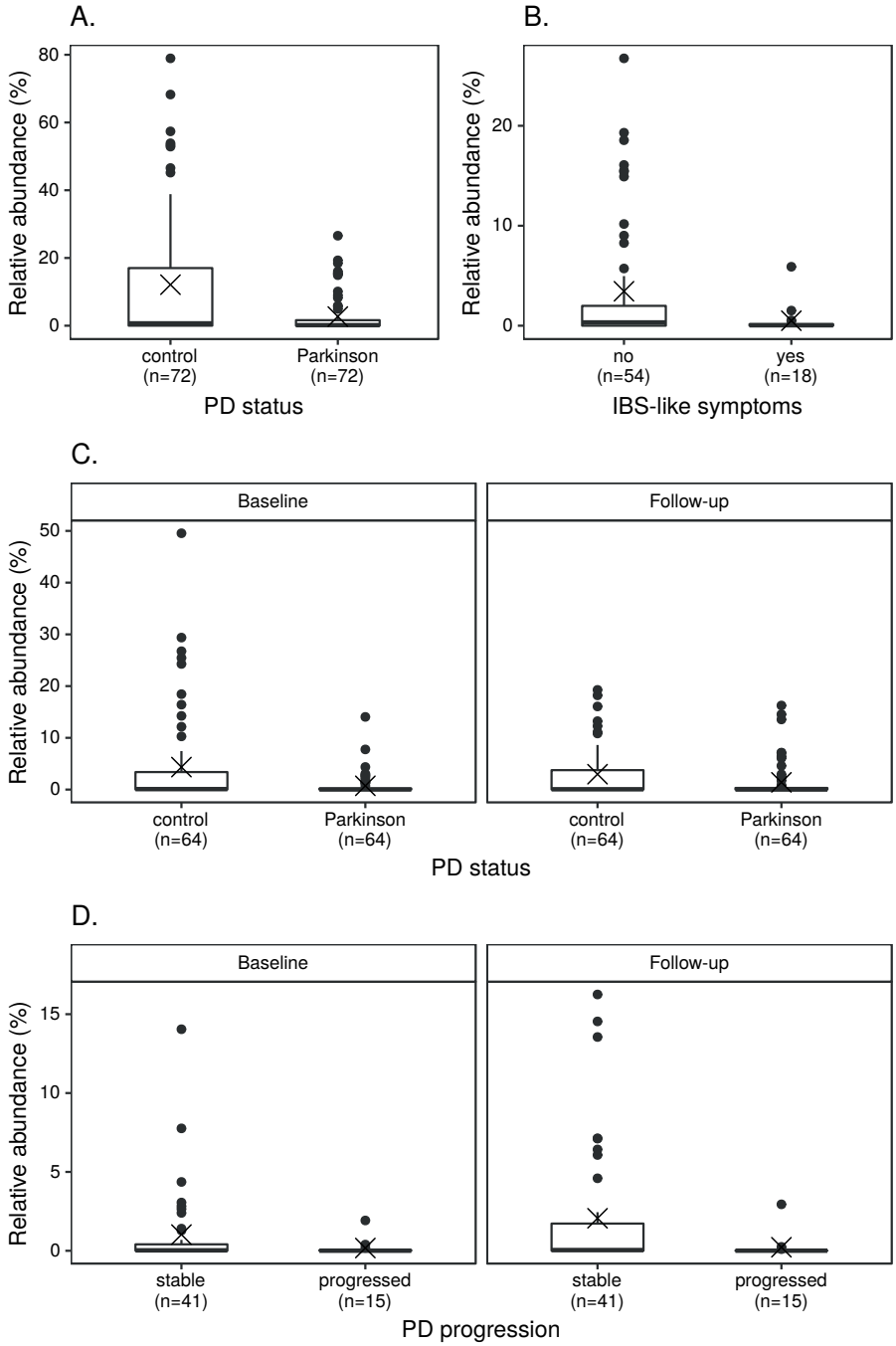
Looking at taxa with multiple comparison corrected $p < 0.05$ (Figure 4.2A), Metastats gave the longest list of OTUs (469), while the list from DESeq2 was the shortest (14). On family level (Figure 4.2B), the original Metastats list was the shortest with its single hit, and metagenomeSeq had the longest list (12). Only the family *Prevotellaceae* and two OTUs, representing the genera *Prevotella* and *Anaerotruncus*, were detected as differentially abundant by all four tools (Table 4.2).

Table 4.2: Additional differential abundance comparisons of gut microbiota at baseline.

| | Adjusted *p*-value | | | | More abundant in | Tools with $p < 0.05$ |
|---|---|---|---|---|---|---|
| **OTU** | **Meta-stats** | **LEfSe** | **meta-genome-Seq** | **DESeq2** | | |
| Otu004 *(Prevotella)* | 0.018 | 0.004 | <0.001 | 0.001 | control | 4 |
| Otu117 *(Anaerotruncus)* | 0.018 | <0.001 | 0.001 | <0.001 | PD | 4 |
| Otu036 *(Roseburia)* | | 0.034 | 0.007 | 0.037 | control | 3 |
| Otu046 *(Lachnospiraceae* incertae sedis) | | 0.002 | 0.009 | 0.017 | control | 3 |
| Otu152 *(Clostridium* sensu stricto) | | <0.001 | <0.001 | <0.001 | control | 3 |
| Otu159 *(Agromonas)* | 0.032 | 0.034 | <0.001 | | PD | 3 |
| Otu300 *(Acetivibrio)* | | <0.001 | <0.001 | 0.037 | control | 3 |
| Otu347 *(Butyrivibrio)* | 0.032 | 0.007 | 0.001 | | control | 3 |
| Otu364 *(Bacteroides)* | | 0.002 | <0.001 | 0.014 | control | 3 |
| Otu407 *(Lachnospiraceae* incertae sedis) | | 0.009 | <0.001 | 0.049 | control | 3 |
| Otu428 *(Anaerovorax)* | 0.032 | 0.023 | <0.001 | | control | 3 |
| Otu484 *(Veillonella)* | | 0.006 | <0.001 | 0.037 | control | 3 |
| **Family** | | | | | | |
| *Prevotellaceae* | 0.031 | 0.045 | 0.007 | <0.001 | control | 4 |
| *Lactobacillaceae* | | 0.045 | <0.001 | <0.001 | PD | 3 |
| *Verrucomicrobiaceae* | | 0.027 | 0.003 | 0.005 | PD | 3 |

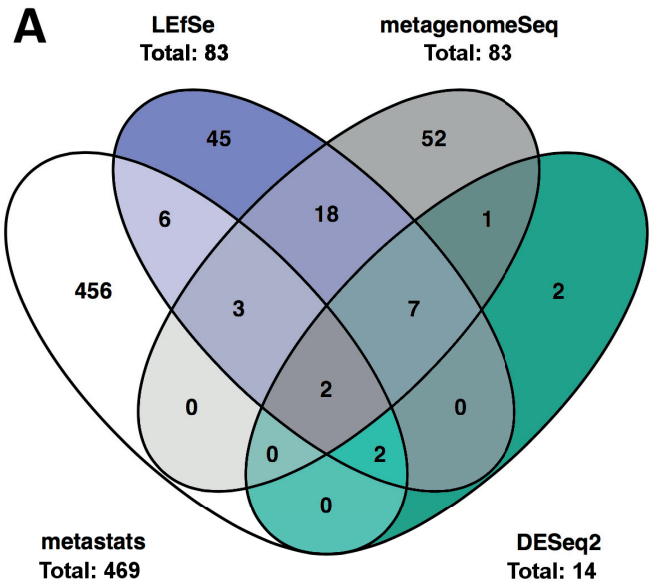
Showing only taxa significant according to three or more methods. Empty cells indicate $p > 0.05$

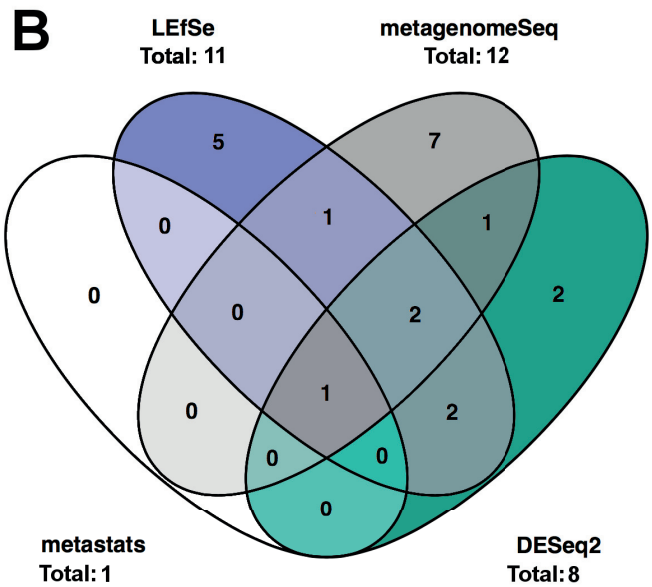
Figure 4.1: Relative abundance of the family *Prevotellaceae*. A. PD vs. controls, baseline (I); B. PD patients with and without IBS-like symptoms (III); C. PD vs. controls at two time points (IV); PD patients with stable and progressed disease (IV).




Box hinges correspond to 1st and 3rd quartiles, and whiskers range from hinge to 1.5 * interquartile range. Samples beyond this range are plotted as points. The × symbol indicates the mean.

Figure 4.2: Venn diagrams of differentially abundant taxa detected at baseline with four different tools. A. OTUs; B. Families.



## 4.2 Nasal, oral and gut data and confounding factors

### 4.2.1 Nasal microbiota (II)

Similarly to the baseline gut study, the nasal microbiota comparisons were a first attempt to see whether we could find any differences in the nasal microbial communities of PD patients and control subjects. We found no differences in alpha or beta diversity between the groups. The differential abundance comparisons were performed with a DESeq2 model including five confounders: DNA extraction batch, sex, age, history of non-major ear, nose or throat (ENT) surgery, and the score for Sniffin' Sticks, a test measuring the sense of smell. The list of differentially abundant taxa for PD status consisted of one taxon on each of the levels explored: the family *Flavobacteriaceae*, the genus *Marmoricola*, and an OTU of the genus *Staphylococcus* (Table 4.3A; II: Supplementary table 5).

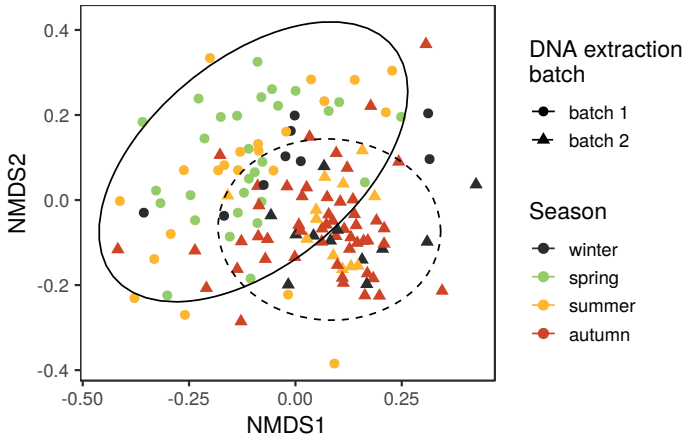
Considering the confounding variables included in differential abundance comparisons, DNA extraction batch was associated with the largest number of differentially abundant taxa: 41 families, 59 genera and 70 OTUs (II: Table 2). The extraction batch overlapped with the season of sample collection, so that most samples collected in spring or summer were handled in the first batch, and samples collected in autumn or winter in the second batch. Extraction batch was also the variable associated with the most notable beta diversity difference ($p < 0.001$, $R^2 = 0.036$), and the beta diversity difference between seasons was close to significant (unpublished; $p = 0.063$, $R^2 = 0.034$; Figure 4.3).

### 4.2.2 Oral microbiota (II)

The mouth was the third anatomic site that we chose for contrasting the microbial communities of PD patients and control subjects. We found no alpha diversity differences between the two groups, but there was a significant difference in beta diversity ($p = 0.0138$, $R^2 = 0.0113$). When testing for confounders, we also found a community difference for sex ($p = 0.0494$, $R^2 = 0.0098$).

The confounders included in the differential abundance comparisons were DNA extraction date, sex, age, history of minor ENT surgery, alcohol consumption (units per week), SDQ score, and SCS-PD score. In line with the beta diversity results, the two variables associated with the largest number of differentially abundant taxa were PD status (11 families, 10 genera and 25 OTUs) and sex (2 families, 10 genera and 16 OTUs) (II: Table 2). The differentially abundant genera detected for PD status were *Haemophilus, Neisseria, Gemella, Rothia, Granulicatella* and *Kingella*, which were more abundant in control subjects, and *Veillonella, Prevotella, Moryella* and *Solobacterium*, which were more abundant in PD patients (Table 4.3B; II: Supplementary table 2).

Figure 4.3: NMDS ordination of nasal microbiota showing the sampling season and DNA extraction batch.




Ellipses represent 95% confidence levels.

Table 4.3: Differential abundance results of article II. A. Nasal microbiota (three taxonomic levels); B. Oral microbiota (showing only genera).

**A.**

| Taxon | Mean relative abundance (%) | | Prevalence in samples (%) | | *p*-value | |
|---|---|---|---|---|---|---|
| | control | PD | control | PD | Unadjusted | Adjusted |
| Otu0006 *(Staphylococcus)* | 5.10 | 5.73 | 97.01 | 97.10 | <0.001 | <0.001 |
| Genus *Marmoricola* | 0.04 | 0.01 | 19.40 | 7.25 | <0.001 | 0.018 |
| Family *Flavobacteriaceae* | 0.19 | 0.13 | 67.16 | 57.97 | <0.001 | 0.011 |

**B.**

| Genus | Mean relative abundance (%) | | Prevalence in samples (%) | | *p*-value | |
|---|---|---|---|---|---|---|
| | control | PD | control | PD | Unadjusted | Adjusted |
| *Haemophilus* | 7.32 | 5.23 | 100.00 | 97.22 | 0.003 | 0.021 |
| *Neisseria* | 6.19 | 2.97 | 100.00 | 97.22 | <0.001 | <0.001 |
| *Veillonella* | 3.40 | 4.87 | 100.00 | 100.00 | <0.001 | 0.005 |
| *Gemella* | 2.95 | 2.69 | 100.00 | 100.00 | 0.009 | 0.047 |
| *Prevotella* | 2.02 | 3.93 | 100.00 | 100.00 | <0.001 | <0.001 |
| *Rothia* | 1.47 | 0.80 | 100.00 | 98.61 | <0.001 | 0.002 |
| *Granulicatella* | 0.67 | 0.60 | 100.00 | 100.00 | 0.006 | 0.034 |
| *Kingella* | 0.29 | 0.22 | 94.74 | 93.06 | <0.001 | <0.001 |
| *Moryella* | 0.02 | 0.03 | 67.11 | 69.44 | 0.002 | 0.012 |
| *Solobacterium* | 0.01 | 0.03 | 57.89 | 69.44 | <0.001 | <0.001 |

### 4.2.3 Gut microbiota and IBS-like symptoms (III)

Since gastrointestinal symptoms, such as constipation, are more common in PD patients than in the general population, we wanted to see if this is true specifically for IBS-like symptoms. Our Rome III questionnaire based assessment suggested that the prevalence of IBS-like symptoms is higher in PD patients than in controls (24.3% vs 5.3%, $p = 0.001$; III: Table 2).

To explore the potential association of gut microbiota and IBS-like symptoms in PD patients, we looked for taxa that are differentially abundant between patients who fill the IBS criteria (n=18) and those who do not (n=54). This was a subgroup analysis of the PD patient data from the baseline gut study (I). We used DESeq2 on the OTU, genus and family levels, both as a simple two-group comparison, and corrected for several potential confounders (hypothyroidism, lactose intolerance, sex, BMI, dopamine agonist use, and Jankovic tremor score).

The results for the models with and without confounders were similar. *Prevotella* and *Prevotellaceae* (Figure 4.1B) were significantly less abundant in patients with IBS-like symptoms according to both models, and the confounder-adjusted model detected one additional taxon, an OTU of the genus *Bacteroides*, which was also less abundant in IBS+ patients (III: Table 4 and Figure 2).

## 4.3 Follow-up gut study: Confounders and two time points

Unlike the other studies in this thesis, which are cross-sectional with one time point, the analyses for the follow-up gut study (IV) included both the baseline data and a second, more recent time point. Due to this, time point was an important additional variable to be considered in all comparisons.

### 4.3.1 Case versus control (IV)

There was no difference in alpha diversity between PD patients and control subjects whether comparing the two time points separately, or merging all the data and disregarding the time point information. Beta diversity comparisons were performed with adonis on three taxonomic levels (OTU, genus and family) in three different ways:

1. PD status + time point
2. PD status + time point + single confounder
3. PD status + time point + selected set of multiple confounders

The single-confounder comparisons (2) were run to define which confounders were significant and should be included in the final model (3). The PD status variable was significant both without confounders and in the model corrected for multiple confounders (IV: Table 8A-C). An additional multiple confounder test was performed with another command, envfit, and this also supported a community difference at all three levels. As for confounding variables, based on the results of single and multiple confounder models, the confounders with the most consistent microbial community effects across comparisons and different taxonomic levels were BMI and Rome III score.

Differential abundance comparisons were performed using three tools: ANCOM, DESeq2, and random forests. Out of these, random forests included no confounder adjustment, ANCOM was adjusted for Rome III and BMI, and DESeq2 included adjustments for the two confounders as well as subject identity. Time points were tested separately with ANCOM and random forests, and in a combined model that included all data with DESeq2. The three approaches gave varying lists of significant taxa (IV: Figure 9, Table 11, Supplementary table S1); for example, the DESeq2 analysis detected only 2 OTUs at baseline and none at follow-up, the ANCOM list included 6 for baseline and 8 for follow-up, while the random forests results had 33 for baseline and 29 for follow-up. A handful of taxa, including *Prevotella* and *Prevotellaceae* (Figure 4.1C; more abundant in controls) and *Bifidobacterium* and *Bifidobacteriaceae* (more abundant in PD patients) overlapped between multiple tools (IV: Figure 9, Table 11).

### 4.3.2 Disease progression (IV)

We found no differences in either alpha or beta diversity in any of the comparisons we performed for the PD progression categories (IV: Table 9). Some confounders, particularly COMT inhibitor medication, stood out as having significant microbial community effects in the beta diversity analyses.

Similarly to the patient to control comparisons, we also used three tools to search for differentially abundant taxa between stable and progressed PD patients. Again, the results varied considerably: ANCOM listed 2 OTUs for follow-up, but no genera or families, and while DESeq2 and random forests detected more taxa, there was little overlap between the results for the two tools, or between the two time points (IV: Table 12, Supplementary table S2). In the DESeq2 results, the genus *Prevotella* differed significantly between groups at both time points, and the family *Prevotellaceae* at follow-up (Figure 4.1D, IV: Figure 11, Supplementary table S2C).

# 5 Discussion

## 5.1 Differential abundance detection: Lessons learned

Through the course of the analyses presented in this thesis, we used a total of six different tools for differential abundance comparisons: Metastats, LEfSe, metagenomeSeq, DESeq2, ANCOM, and an approach based on random forests. Although random forests is a supervised learning algorithm and not a differential abundance detection tool as such, it can also provide information on taxa that are are the best at differentiating between groups of interest, offering an analogous way to label microbes of interest. Most of the publications included in this thesis only made use of a single tool: Metastats for the baseline gut study (I), and DESeq2 for the oral and nasal study (II) and the within-PD gut symptoms study (III). However, we also used three additional tools to re-analyse the baseline data in an unpublished analysis presented in this thesis, and the analyses for the follow-up study included a combination of three different tools (IV).

### 5.1.1 Confounder selection

The tools used in this thesis vary in how flexible they are regarding the possible comparisons, ranging from the simplest cases that only allow two-group contrasts, like Metastats and LEfSe, through those with the possibility for confounder adjustments, like ANCOM 2.0, to the tools where the user can specify complex models with interactions and nested designs, like metagenomeSeq and DESeq2. These differences mean that the features of the tool affect how easy it is to correct for potential confounding variables.

Selecting which confounding variables to correct for in a statistical model is not easy, nor is it evident what is the best way to implement such a correction (Christenfeld et al., 2004; Lu, 2009). There is no established consensus on the most important confounders for studying the human microbiome (Knight et al., 2018). Our subjects were originally matched for age and sex (I), but there are definitely many other factors that could affect subjects' microbial communities, for example BMI (Chen et al., 2016a; Davis et al., 2017; Marchesi et al., 2016; Rothschild et al., 2018), diet (Chen et al., 2016a; Claesson et al., 2012; David et al., 2014; Davis et al., 2017), and medications (Forslund et al., 2015). A far more difficult question is whether the effects of the various non-motor symptoms, such as GI disturbances, can be disentangled from overall disease-related changes in microbiota.

The choices made regarding confounder correction in the four studies included in this thesis were varied, partly because of practical reasons. Since Metastats only allows comparisons of two categorical variables, we complemented the initial differential abundance analysis in the baseline study (I) with GLMs for the main taxa of interest, including multiple variables in the models. This additional analysis offered further support to the Metastats findings, but also revealed some confounding effects.

DESeq2, the most frequently used tool in this thesis, is itself based on GLMs and allows the user to specify as many explanatory variables as they want in their statistical model. In the nasal and oral study (II) we chose to include all potential confounders, leading to long lists of them (five for nasal, seven for oral comparisons). Such an approach could produce

overly conservative results. Nevertheless, in this particular case, the difference between the nasal and oral results was striking, with only a single taxon at the three taxonomic levels flagged as differentially abundant in the nasal data, while the oral comparisons resulted in ten or more hits on each of the three levels. In the PD-only comparisons of IBS-like symptoms (III), we tested both an uncorrected DESeq2 model, and one corrected for six confounding variables. The results of the two models were nearly identical, with the confounder-corrected model suggesting a single additional OTU that was not detected as significant with the uncorrected model.

In the follow-up analysis (IV), we used a different confounder selection strategy than in the previous analyses, namely basing the choices on the results of beta diversity comparisons. The list of variables with minor but significant beta diversity effects was long, and instead of using all of them, we selected the ones that were most consistently significant in comparisons performed at different taxonomic levels and with two different tests. The same confounders were included in both DESeq2 and ANCOM analyses, although the DESeq2 comparisons between patients and controls also included two additional variables: time point and subject identity. Time point was introduced as an interaction variable together with PD status, to evaluate between-time point differences, and subject identity was included to account for the intersubject variability. A further complication was that we wanted to include BMI, but lacked that information for some subjects, and excluding them left an unequal amount of subjects in the patient and control groups. This led to the idea of running the test multiple times in a leave-one-out loop, and considering the average $p$-values as the final result. The results of this more complicated approach still included several genera and families that were significant for the patient-to-control contrast, but surprisingly, only two OTUs at baseline, and none at follow-up. This might reflect the sparsity of the OTU-level count table, which makes the comparisons vulnerable to outlier effects, so that leaving out even one sample can affect the outcome noticeably. Perhaps the lack of OTUs with a significant consensus average $p$-value in this comparison can be considered a cautionary example of how inconsistent OTU-level analyses can be. Performing similar analyses with unique sequence variant data, which is typically even sparser, is not likely to be an improvement.

Finally, aside from the more technical challenges of confounder correction, some important confounding factors may be missing because we did not measure them. For example, we did not collect dietary data for the first time point, and therefore could not correct for dietary effects in the first three studies (I-III), which is a major study limitation in them. The follow-up study (IV) included comparisons of FFQ-based dietary data, with results that overall did not suggest dietary differences between PD patients and control subjects, but it must be noted that the number of subjects was small for this type of analysis. FFQs, which rely on the subjects' ability to accurately report their typical dietary intakes of specific food items, are prone to biases and errors (Stumbo, 2013), and the limited statistical power in our study may have masked some dietary effects.

### 5.1.2 Contrasting tools

Ease of use is often an important factor when researchers consider which biological data analysis tool to choose (Boulesteix et al., 2018), and is something that cannot be taken for granted. In fact, a recent preprint publication that tested 24 490 tools found that as many as 28% of them could not even be installed (Mangul et al., 2018). While all tools used in this thesis were fairly easy to take into use, they do have differences in usability. Since Metastats is included in mothur, the tool used for microbiota sequence analysis in all of the publications in this thesis, it could be run easily at the end of the sequence analysis

pipeline, only requiring a simple additional file to designate sample groupings. LEfSe was run on an online platform, which was equally simple to use, although it did require setting up the data in a specifically organized table beforehand; an option of running LEfSe in mothur is also available. MetagenomeSeq, DESeq2 and ANCOM are all implemented in R. MetagenomeSeq and DESeq2 are available through the Bioconductor repository (Huber et al., 2015), which makes their installation easy for anyone familiar with R. Both also have good documentation. While DESeq2 is originally a tool for differential gene expression analysis of RNA-seq data, instructions for using it for microbiota analyses are available from the authors of the phyloseq microbial ecology R package, and are simple to follow. ANCOM is distributed as a standalone script. We used the latest version available at the time; this was a version called ANCOM 2.0 which was available on the author's website, and the scripts needed manual adjustments to work at all, so in this sense, it was the worst performer. The random forests approach was the most complicated one, involving three different R packages, but as it was a custom-written implementation for this analysis, not a ready-to-use tool, it cannot be directly compared to the five others.

In addition to practical implementation, the tools vary in the amount of information they provide regarding the results of the analysis, influenced both by the statistical backgrounds and by different choices of each tool's creators. Metastats gives group means, variances and standard errors, and adjusted and unadjusted *p*-values for each taxon. LEfSe's output table has the log of the highest class average, the linear discriminant analysis (LDA) effect size, and *p*-value. There are multiple ways to access the results of metagenomeSeq's tests, such as the fitZig function which was used here, with a typical table including a value for the coefficient of interest (the specific unit of which depends on the target variable), *p*-value, and adjusted *p*-value. DESeq2 reports the overall mean count for each taxon, the unadjusted and adjusted *p*-value, the Wald statistic used to calculate the *p*-value, the log2 fold change (between groups for categorical variables, per one unit change for continuous numerical ones), and the standard error for the log2 fold change. The first version ANCOM only gave a list of taxa flagged as significant and a W-statistic, which was not explained further in the documentation; the version 2.0 used here produces a larger output table with the W-statistic and a list stating whether or not each taxon is significant for a series abundance cutoffs. Since there is no standardized implementation for random forests in microbiota analyses, the output will be defined by the specific packages used and the analyst's choices. As demonstrated by the descriptions above, the amount of documentation for the tools varies just as their output formats do, which can lead to confusion regarding the exact meaning of the results. The variation in outputs adds to the challenges of presenting and comparing the differentially abundant taxa detected by different tools.

Considering the results of the comparisons in this thesis that were performed with multiple tools, the unpublished reanalysis of the baseline gut data produced a single family (*Prevotellaceae*) and two OTUs (representing the genera *Prevotella* and *Anaerotruncus*) that all four tools (Metastats, metagenomeSeq, LEfSe and DESeq2) agreed on, and an additional two families and ten OTUs supported by three of them. These comparisons were not corrected for confounding variables. The amounts of hits listed by the different tools were also quite varied, ranging from the 469 OTUs detected by Metastats to 14 detected by DESeq2. The exact same baseline stool samples were compared using three tools (ANCOM, DESeq2 and random forests) after re-sequencing for the follow-up study. All three only agreed on a single taxon: the family *Prevotellaceae*. Again, the amounts of detected taxa also varied, with 33 OTUs significant according to the random forests approach, 6 according to ANCOM, and 2 according to the DESeq2 analysis. In these comparisons, ANCOM and DESeq2 included confounder correction. Regarding the follow-up time point,

the results of the three tools were just as varied, with the genus *Prevotella* as the only taxon with a consensus from all three; the family *Prevotellaceae* was supported by DESeq2 and ANCOM, but not random forests. On one hand, our combined results from six differential abundance detection tools highlight the inconsistencies between them. On the other, they seem to indicate that when a signal is particularly strong, such as the differences between *Prevotellaceae* and *Prevotella* in our data, it can be successfully detected with many different tools.

Similarly to the practical example provided by the analyses in this thesis, past benchmarking studies have reported widely varying results from different tools (Hawinkel et al., 2017; Thorsen et al., 2016; Weiss et al., 2017). Many tools may have a high false positive rate, while more conservative ones risk the inverse problem of having low sensitivity (Hawinkel et al., 2017). Using multiple tools can be one way to overcome the limitations of any single one, although it comes with its own set of problems: if only the overlapping consensus is considered significant, there is a risk of overlooking important taxa that are not detected by the less sensitive approaches. There is also no consensus for how to best report results from multiple tools, tempting researchers to cherry-pick those that look the best while ignoring the tools that report no significant hits (Boulesteix et al., 2018). Studies that rigorously report the full result tables from many different tools risk becoming impenetrable to the non-expert reader, a challenge that we faced when presenting the analyses of the follow-up article (IV). Still, based on the experience gained from the analyses in this thesis, tackling these challenges is a better alternative than trusting the more easily reported and interpreted results of a single tool.

New differential abundance detection tools are being published constantly. At least six publications presenting new methods for identifying taxa of interest in microbial survey data have came out since the analyses of the most recent article in this thesis (Chai et al., 2018; Koh et al., 2017; Morton et al., 2017; Rivera-Pinto et al., 2018; Tang and Chen, 2018; Zhang et al., 2017). Such publications typically include comparisons contrasting them with older tools, but these are not standardized between studies, and may not give a realistic picture of the tool's performance due to author bias (Boulesteix et al., 2018). Installing and learning a new tool may involve a considerable amount of work, which leads to a tendency for researchers to keep using the tools they already know, unless there are particularly strong reasons for switching to a new one (Boulesteix et al., 2018). There is a definite need for frequent, independent benchmarking studies to explore the performance of the various tools as they become available, preferably with standardized test data that could be used to reliably compare them against tools that are already well established in the field.

## 5.2 Nasal and oral microbiota in Parkinson's disease: Case closed?

### 5.2.1 Nasal microbiota and practical challenges

Our analyses of nasal microbiota (II) did not suggest a strong difference between the PD patients and control subjects: the alpha diversity was similar, and there was no significant difference in beta diversity, while there was a strong difference in relation to extraction batch. The extraction batches were also related to sample collection season, which could be an important confounder for nasal microbiota because of environmental factors such as changes in temperature and humidity (Bogaert et al., 2011); due to the collinearity of the two variables, we could not distinguish between potential seasonal effects and technical batch

variation. This highlights the importance of technical considerations, such as randomization in sample handling.

Our differential abundance comparisons picked up a single taxon on each of the three levels tested (OTU, genus and family). The OTU was more abundant in patients and belonged to the genus *Staphylococcus*. We attempted to further explore *Staphylococcus* strains using a more detailed approach, oligotyping (Eren et al., 2013), but this revealed no differences in the abundances of oligotypes between patients and controls. The genus *Marmoricola* was more abundant in controls. This genus, which was originally described based on a species isolated from a marble statue, only contains environmental taxa (Kim et al., 2015b; Urzi et al., 2000) and is not a known human colonizer, raising suspicions regarding its origin and biological significance. Three known laboratory contaminant genera (*Halomonas, Shewanella* and *Ralstonia*) were trimmed from the nasal data prior to analysis, but it is possible that other contaminant taxa remained; these are particularly common in low-biomass samples (Salter et al., 2014), of which nasal swab samples are an example. The family *Flavobacteriaceae*, which was also more abundant in controls, contains some human-associated taxa, such as the pathogens *Capnocytophaga canimorsus* and *Elizabethkingia meningoseptica*, the former related to dog bites, the latter an opportunistic pathogen which mainly causes infections in young children (McBride, 2014). However, only the family was detected as significant, not hinting at a difference in the abundance of any specific pathogen.

One other study (Heintz-Buschart et al., 2018), published after ours, has explored the nasal microbial communities of PD patients and controls, and subjects with idiopathic rapid eye movement sleep behavior disorder, a condition linked with a high risk of later developing PD (Howell and Schenck, 2015; Iranzo et al., 2014; Schenck et al., 2013). Their sampling approach was different from ours, using nasal lavage fluid instead of swabs, and they only analyzed nasal data at the family level due to the sparsity of the data – many of the OTUs were only present in a handful of subjects. This could be either due to the high interindividual variation in nasal microbial communities (Biswas et al., 2015), to the difficult sample material leading to failure to detect low-abundance taxa, or both. Heintz-Buschart et al. (2018) found no beta diversity differences, and while they did detect a total of eight families that were differentially abundant between PD patients and control subjects, one was also related to L-dopa use, suggesting that it might be linked to the medication rather than disease status. The family *Flavobacteriaceae* was not on their list of significant families. They surmised that nasal microbiota do not seem promising as a potential source of biomarkers for PD, a conclusion that seems in line with our results.

Based on these two studies, studying nasal microbiota with an amplicon sequencing approach may not be particularly useful for furthering our understanding of PD pathogenesis, or discovering biomarkers or new treatment modalities. One factor to consider regarding studies such as these is that the olfactory bulb, the structure affected by early alpha-synuclein pathology, is not reachable by non-invasive sampling methods. Then again, if an external agent affecting the disease pathogenesis enters through the nasal epithelium, as suggested in Braak's two-hit hypothesis (Hawkes et al., 2007), that agent could be present in more easily reachable anatomic structures. Still, even if it exists, it could be a transient visitor, not a permanent colonizer, making it detectable at only some very specific window in time. Regardless of sampling method, the study of nasal microbiota will be complicated by the technical challenges caused by the low amount of bacterial DNA in samples. While it cannot be said that the two so far published studies entirely rule out the possibility of nasal microbial involvement in PD, planning future research into the topic should seek for an alternative approach to ensure that the sampling will capture all relevant microbes.

### 5.2.2 Oral microbiota warrant further study

Contrasting the results of our nasal and oral microbiota analyses reveals a striking difference. While the alpha diversity was similar in PD patients and control subjects at both locations, beta diversity comparisons showed a significant difference in the oral microbial communities of the two groups, and there were many differentially abundant taxa between them as well, ranging from common oral genera like *Haemophilus, Neisseria, Veillonella* and *Prevotella* (the first two more abundant in controls, the others in PD) to low-abundance OTUs. All OTUs belonging to families and genera that were differentially abundant consistently followed the same trends; for example the family *Prevotellaceae*, the genus *Prevotella* and four *Prevotella* OTUs were more common in PD patients, and the family *Neisseriaceae*, the genus *Neisseria* and six *Neisseria* OTUs more common in control subjects.

There is no clear pattern regarding the pathogenicity of the differentially abundant taxa. Most of the bacterial genera on the list are known to cause infections at least occasionally, and while some of them are more abundant in patients than in controls, others are decreased in patients. Of course, there is no clear-cut definition for which taxa are pathogenic and which are not; many oral taxa that are generally considered commensals can be opportunistic pathogens, particularly in immunocompromised subjects (Belibasakis and Mylonakis, 2015; Dahlen, 2009). Since our taxonomic information is limited to the genus level, identifying species or strains of varying clinical significance is not possible. Overall, we did not see an overabundance of potential pathogens in PD patients compared to controls. Given that there are conflicting reports on whether PD patients have worse dental health than the general population or not, this is perhaps not surprising (Rozas et al., 2017). Our clinical data collection did not include a dental examination, so we could not evaluate the situation in our subjects in any detail. Performing future studies of PD and oral microbiota in collaboration with dentists could help provide more accurate information on dental health parameters in the analyses. Additionally, there are various microenvironments in the oral cavity, and our approach did not target any specific one; the sampling was done with swabbing both the sublingual and buccal mucosa. This will give a general impression of a subject's oral microbiota rather than an accurate view of a specific microbial community, somewhat similarly to saliva sampling (Shi et al., 2018; Simón-Soro et al., 2013). More detailed characterization of different oral niches could reveal details about the differences between PD patients' and controls' microbiota.

Although oral and gut studies are typically separate, with researchers specializing in one location or the other, the two sites are both part of the GI tract, two ends of a single continuous tube. The distribution of bacterial taxa typically detected in stool is very different from that of oral samples (Gilbert et al., 2018; Grice and Segre, 2012; Human Microbiome Project Consortium, 2012). However, one study contrasting the communities of different parts of the body suggested that the composition of oral microbiota, particularly from salivary samples, is associated with stool microbiota composition; for example, high oral levels of *Prevotella* seem to go hand in hand with high gut levels (Ding and Schloss, 2014). Experimental studies in mice have also explored oral microbes' ability to colonize the gut; Seedorf et al. (2014) found that human tongue microbes are not very successful at colonizing the mouse gut, while Atarashi et al. (2017) discovered that *Klebsiella* strains isolated from human saliva can colonize the mouse gut and activate an immune response.

In our data, oral and gut *Prevotella* abundances were not correlated; overall, the genus was more abundant in PD patients' oral samples, but less abundant in their stool samples than in those of control subjects. This seemingly contradictory situation can be easily explained by the *Prevotella* OTUs representing different species, as they are likely to do: they exist in

different niches, and it has been shown that *Prevotella* isolates from different parts of the body have notable differences in their genetic repertoire (Gupta et al., 2015). We searched for overlapping OTUs in the gut and oral datasets in an unpublished analysis (data not shown), and found very few that were shared between the two locations. However, this comparison was based on a resequencing of the baseline gut samples that was not used for the final analyses due to quality issues. The most recent resequencing, which was better quality-wise, used primers targeting a region different from the oral analyses (V3-V4 instead of V1-V3), which complicates comparative analyses. For better comparability, the oral and gut samples should be extracted, amplified and sequenced simultaneously. Such a study could be of interest not only for PD research but also to explore whether the oral microbiota composition in fact reflects the gut community.

To our knowledge, no studies other than ours have explored PD and oral microbiota with a 16S rRNA gene amplicon sequencing approach. The potential links between oral health and oral microbiota have been studied more in Alzheimer's disease, and the connection of oral health and neurodegeneration is perhaps stronger for AD than for PD, including for example a large cohort study that found an increased risk of AD in subjects with chronic periodontitis (Chen et al., 2017). There are several studies suggesting the presence of microbial DNA or molecules such as lipopolysaccharide in the brains of subjects with AD (Emery et al., 2017; Poole et al., 2013; Zhan et al., 2016), leading researchers to theorize that bacteria from anatomic locations including the mouth could be displaced into the brain, contributing to the disease process (Pritchard et al., 2017). Up to date, there have not been any amplicon sequencing based studies directly addressing the relationship of AD and oral microbiota. Due to the potential parallels between AD and PD – two common neurodegenerative diseases with a suspected connection to microbiota – one future approach could be a combined analysis of oral microbes in both.

Our study is a first foray into exploring the relationship of PD and oral microbiota and a stepping stone for further studies. Overall, the oral health of PD patients is a somewhat underexplored aspect of the disease with notable repercussions on their quality of life (Rozas et al., 2017). Since our results clearly show that there are differences between the oral bacterial communities of control subjects and PD patients, the disease-related changes in oral microbiota definitely warrant more research in the future.

## 5.3 The evolving story of gut microbiota in Parkinson's disease

### 5.3.1 Is there a consensus?

Our baseline study (I) was the first 16S rRNA gene amplicon sequencing based publication of Parkinson's disease and gut microbiota. In the following years, a number of other research groups from around the world have reported results using amplicon sequencing (Table 5.1; Heintz-Buschart et al., 2018; Hill-Burns et al., 2017; Hopfner et al., 2017; Keshavarzian et al., 2015; Li et al., 2017; Lin et al., 2018; Petrov et al., 2017; Qian et al., 2018b), shotgun metagenomics (Bedarf et al., 2017), or other DNA-based methodology (Hasegawa et al., 2015; Unger et al., 2016). All these studies have a case-control design, with the number of subjects ranging from 24 patients and 14 controls (Li et al., 2017) to 197 patients and 130 controls (Hill-Burns et al., 2017). They represent different populations across the continents: Germany (Bedarf et al., 2017; Heintz-Buschart et al., 2018; Hopfner et al., 2017; Unger et al., 2016), the United States (Hill-Burns et al., 2017; Keshavarzian et al., 2015), Russia

(Petrov et al., 2017), Japan (Hasegawa et al., 2015), and China (Li et al., 2017; Lin et al., 2018; Qian et al., 2018b). Aside from the methodological and geographical differences, the studies have used varying selection criteria for their patients as well as their control subjects, some choosing to recruit spouses (Hasegawa et al., 2015; Lin et al., 2018; Qian et al., 2018b) or a mix of spouses and unrelated healthy subjects (Hill-Burns et al., 2017), hoping that this will help control for the effects of diet and lifestyle factors. Most, but not all, studies have used age-matched controls.

Even if one only focuses on the 16S rRNA gene amplicon sequencing studies of PD and gut microbiota, there are many technical differences, such as different DNA extraction kits, sequencing platforms and target regions (Table 5.1). There may also be major differences in sample storage and handling; one potential source of bias in our follow-up study (IV) is that the baseline stool samples were thawed twice (first for sequencing at baseline for I, then for resequencing at follow-up for IV), with approximately two years of storage time between the time points. Therefore it is reassuring that we did not detect any notable microbial community differences between the twice-thawed baseline samples and the freshly collected and analyzed follow-up samples.

The statistical analyses also differ between studies. The confounder correction approaches range from the simplest case of contrasting patients to control subjects with no confounders (Keshavarzian et al., 2015; Li et al., 2017) to complicated modeling and exploration of additional variables (Hill-Burns et al., 2017). The studies use a variety of differential abundance detection tools: the Kruskal-Wallis test (Bedarf et al., 2017; Keshavarzian et al., 2015), Metastats (Li et al., 2017; Petrov et al., 2017), LEfSe (Lin et al., 2018; Qian et al., 2018b), a combination of 1000-fold Monte-Carlo simulation Wilcoxon test and quasi-Poisson regression (Hopfner et al., 2017), Kruskal-Wallis, ANCOM and glmmADMB (Hill-Burns et al., 2017), and ANCOM and DESeq2 (Heintz-Buschart et al., 2018). An additional challenge in comparing the results of these studies is that their analyses target different taxonomic levels, some focusing on families like our baseline study, others including multiple levels. The abundances of non-significant taxa are typically not reported.

Taking into account both population variation and all the differences in methodological choices, it is not unexpected that the results of the studies exploring gut microbiota and PD are inconsistent. Reassuringly, there are also patterns that remain similar across studies. Regarding alpha diversity, most studies have found no differences; the exceptions are Keshavarzian et al. (2015), who detected an increased diversity in PD patients with three metrics on phylum level and one on genus level; Petrov et al. (2017), who found a decreased Chao1 richness in PD patients; Lin et al. (2018), who state that they saw no alpha diversity difference while simultaneously reporting a significant $p$-value for Chao1 (they do not mention whether it was higher in patients or controls); and Qian et al. (2018), who found a significantly increased diversity in PD patients with five different metrics. To summarize, including our two studies (I, IV), four out of ten found no difference, two did not report alpha diversity results, one discovered a decreased richness in PD, and three detected a higher diversity in PD with varying metrics. There does not seem to be a consistent pattern regarding alpha diversity. On the other hand, every amplicon sequencing based study so far has detected a difference in beta diversity (Table 5.1), although they have used varying metrics and tests, giving strong support to our findings that the gut bacterial communities differ between patients and controls.

The results of the various PD and gut microbiota publications vary the most when it comes to the differentially abundant taxa. Considering both the differences in methodological details between studies, and the differences between tools, as seen earlier in this thesis, this is not particularly surprising. The tools used by more than one amplicon sequencing

Table 5.1: Published 16S rRNA gene amplicon sequencing based studies of Parkinson's disease and gut microbiota.

| Reference | n (PD/ controls) | Subject nationality | DNA extraction kit | Seq platform | Target region | Alpha diversity differences | Beta diversity differences | Differential abundance method(s) | Taxonomic levels tested |
|---|---|---|---|---|---|---|---|---|---|
| **I** | 72/72 | FI | PSP Spin Stool DNA Plus Kit | 454 | V1-V3 | none | sign. | metastats | F |
| **Keshavarzian et al. 2015** | 38/34 | US | Fast-DNA Spin Kit for Soil | MiSeq | V4 | higher in PD | sign. | K-W | G, F, P |
| **Petrov et al. 2017** | 89/66 | RU | in-house protocol | MiSeq | V3-V4 | lower in PD | sign. | metastats | S, G |
| **Hopfner et al. 2017** | 29/29 | DE | PowerSoil Kit | MiSeq | V1-V2 | none | sign. | MC Wilcoxon, quasi-Poisson regression | F |
| **Hill-Burns et al. 2017** | 197/ 130 | US | not specified | MiSeq | V4 | not reported | sign. | K-W, ANCOM, glmm-ADMB | O, G, F |
| **Li et al. 2017** | 24/14 | CN | TIANamp stool DNA kit | MiSeq | V3-V5 | none | different (no *p*-value given) | metastats | G, F |
| **Heintz-Buschart et al. 2018** | 26/38 (+20 iRBD) | DE | Qiagen AllPrep | MiSeq | V4 | not reported | sign. | ANCOM, DESeq2 | O, G, F, P |
| **Lin et al. 2018** | 75/45 | CN | Huirui DNA kit | HiSeq | V4 | sign. (direction not given) | sign. | LEfSe | O, G, F |
| **Qian et al. 2018** | 45/45 | CN | QIAamp DNA Stool Mini Kit | MiSeq | V3-V4 | higher in PD | sign. | LEfSe | G |
| **IV** | 64/64 | FI | PSP Spin Stool DNA Plus Kit | MiSeq | V3-V4 | none | sign. | ANCOM, DESeq2, random forests | O, G, F |

iRBD: idiopathic REM sleep behavior disorder
Subject nationalities: FI : Finland; US: United States; RU: Russia; DE: Germany; CN: China
sign.: statistically significant
K-W: Kruskal-Wallis test
MC Wilcoxon: Monte-Carlo simulation Wilcoxon test
Taxonomic levels: O: OTU; S: species; G: genus; F: family; P: phylum

Table 5.2: Bacterial taxa detected as differentially abundant in three or more publications.

| | Increased in PD | | Decreased in PD | | |
|---|---|---|---|---|---|
| | Statistically significant | Not significant | Statistically significant | Not significant | Supported at follow-up time point (IV) |
| **Phylum** | | | | | |
| *Actinobacteria* | 1 | 0 | 2 | 0 | not tested |
| *Bacteroidetes* | 1 | 1 | 2 | 0 | not tested |
| *Firmicutes* | 1 | 0 | 2 | 0 | not tested |
| *Verrucomicrobia* | 2 | 1 | 0 | 0 | not tested |
| **Family** | | | | | |
| *Bifidobacteriaceae* | 2 | 1 | 0 | 1 | increased in PD (A, R) |
| *Enterobacteriaceae* | 2 | 0 | 0 | 1 | no |
| *Enterococcaceae* | 2 | 0 | 1 | 0 | no |
| *Erysipelotrichaceae* | 2 | 0 | 1 | 1 | no |
| *Lachnospiraceae* | 0 | 0 | 3 | 1 | no |
| *Lactobacillaceae* | 2 | 1 | 2 | 0 | increased in PD (R) |
| *Prevotellaceae* | 0 | 0 | 2 | 4 | decreased in PD (A, D) |
| *Verrucomicrobiaceae* | 4 | 3 | 0 | 0 | no |
| **Genus** | | | | | |
| *Akkermansia* | 4 | 0 | 0 | 0 | no |
| *Bifidobacterium* | 3 | 1 | 0 | 1 | increased in PD (A, R) |
| *Blautia* | 0 | 0 | 3 | 0 | no |
| *Eubacterium* | 1 | 0 | 0 | 2 | no |
| *Faecalibacterium* | 0 | 0 | 3 | 1 | no |
| *Lactobacillus* | 3 | 0 | 1 | 0 | increased in PD (R) |
| *Prevotella* | 0 | 0 | 1 | 3 | decreased in PD (A, D, R) |

A: ANCOM; D: DESeq2; R: Random forests

The summarized values of this table include results of all studies using any DNA-based methodology: Keshavarzian et al., 2015; Hasegawa et al., 2015; Unger et al., 2016; Bedarf et al., 2017; Hill-Burns et al., 2017; Hopfner et al., 2017; Li et al., 2017; Petrov et al., 2017; Heintz-Buschart et al., 2018; Lin et al., 2018; Qian et al., 2018b; and article I of this thesis.

based publication (Table 5.1) are Metastats (three studies), the Kruskal-Wallis test (two studies), ANCOM (three studies), DESeq2 (two studies) and LEfSe (two studies). A large number of taxa are only mentioned in the results of one study and not seen again in any of the others. Combining all taxa that have been mentioned more than twice, including those that differ numerically between groups without being statistically significant, gives a shortlist of 7 genera, 8 families and 4 phyla (Table 5.2). Some signals seem fairly consistent: *Blautia*, *Lachnospiraceae*, *Faecalibacterium, Prevotella* and *Prevotellaceae* were listed as decreased in PD by all the publications that mentioned them. Out of the taxa that are more abundant in PD, *Akkermansia, Verrucomicrobiaceae* and *Verrucomicrobia* were consistent in all publications. *Bifidobacteriaceae, Bifidobacterium, Enterobacteriaceae* and *Lactobacillus* had an increased abundance in all but one publication that mentioned them, and for the first three, the only contradictory mention was not statistically significant. There are also several taxa where the situation is not as clear: for example, the main bacterial phyla in the gut, *Bacteroidetes* and *Firmicutes*, have been reported as both significantly increased and decreased in PD, as has the phylum *Actinobacteria*, although it must be noted that many

of the publications, including ours, did not perform phylum level comparisons.

Our most recent comparisons for the follow-up time point (IV) only supported a few of the taxa from the summary table (Table 5.2), mainly *Bifidobacterium* and *Bifidobacteriaceae*, *Prevotella* and *Prevotellaceae*, and *Lactobacillus* and *Lactobacillaceae*. If one were to suggest a consensus of taxa with an altered abundance in PD patients, it could consist of these three genera and their corresponding families, as well as the genus *Akkermansia* and its parent family, *Verrucomicrobiaceae*. The last two taxa were not significant in our latest comparisons, but have been detected in several previous publications. A recent review article that attempted to summarize the findings of all PD and gut microbiota publications to date ended up with a similar final list: increased *Lactobacillus, Bifidobacterium, Verrucomicrobiaceae* and *Akkermansia*, and decreased *Faecalibacterium, Coprococcus, Blautia, Prevotella* and *Prevotellaceae* (Gerhardt and Mohajeri, 2018). Still, even considering the taxa with the most support from the studies published so far, only three or four out of eleven publications have agreed on their statistical significance.

Out of the suggested consensus taxa detected as differentially abundant in our studies, *Bifidobacterium* and *Lactobacillus* strains are commonly used probiotics (Sanders et al., 2017). There are several examples of beneficial effects of bifidobacteria and lactobacilli from some of the first microbiome–gut–brain axis studies in rodents: for example, *B. infantis* alleviated the behavioral changes in GF mice, which have an altered stress response (Sudo et al., 2004), and in a rat maternal separation model of depression (Desbonnet et al., 2010), while *L. rhamnosus (JB-1)* reduced stress and anxiety in mice subjected to several stress-inducing tests (Bravo et al., 2011). This makes their increased abundance in PD somewhat surprising. On the other hand, probiotic effects are typically strain-specific (Sanders et al., 2017), and bacteria from both genera can occasionally cause infections, particularly in very young, elderly or immunocompromised subjects (Esaiassen et al., 2017; Kochan et al., 2011; Pararajasingam and Uwagwu, 2017).

Many studies have demonstrated that fiber consumption and plant-based diets are associated with high levels of *Prevotella* in the gut (De Filippo et al., 2010; De Filippis et al., 2016; Kovatcheva-Datchary et al., 2015). The genus was also specifically connected to improved glucose tolerance in subjects undergoing a barley kernel-based dietary intervention (Kovatcheva-Datchary et al., 2015), and a high *Prevotella*-to-*Bacteroides* ratio predicted better success at losing body fat on a high fiber diet (Hjorth et al., 2018). Changes in *Prevotella* abundances have also been reported in relation to various, seemingly unrelated unhealthy states: increased abundance in subjects with HIV (Dillon et al., 2015) and new-onset rheumatoid arthritis (Scher et al., 2013; Scher et al. 2016), and decreased in subjects with a high risk for type 1 diabetes (Alkanani et al., 2015), men with chronic prostatitis/chronic pelvic pain syndrome (Shoskes et al., 2016), and children with ASD (Kang et al., 2013). The case of ASD is particularly intriguing, since an open-label microbiota transfer therapy intervention, which improved both behavioral and GI symptoms, also resulted in an increased abundance of *Prevotella* (Kang et al., 2017). Yet, in spite of its association with many different medical conditions, a general synthesis for the role that this genus plays in human health and disease is still lacking (Ley, 2016). It has been suggested that a more detailed understanding of *Prevotella* strains and their wide genetic repertoire (Gupta et al., 2015) could be the key to explaining their significance (Ley, 2016). This is equally true in the context of PD, and for the other taxa of interest as well: explaining the detected changes in abundances will require deeper characterization of the bacterial strains in question, as well as further basic research into the functions of these taxa in the human gut ecosystem.

### 5.3.2 Unsolved challenges and future prospects

There are many criticisms that can be made regarding the study of gut microbiota through targeted 16S rRNA gene sequencing of fecal samples, and all are relevant to performing such analyses in the context of Parkinson's disease. Some of them were outlined in the Introduction of this thesis, including the many potential biases related to the laboratory workflow and the bioinformatic and statistical analyses, and the particular challenges of microbiota data, such as zero-inflation, sparsity and compositionality. In addition to these practical questions, there are deeper ones. One that was already mentioned is whether fecal samples meaningfully reflect the biologically relevant microbial communities of the human gut. Furthermore, the amplicon sequencing approach does not enable accurate identification of the bacteria down to species or strain level, and detects all bacterial DNA in the samples, not only that which originates from viable cells. It also does not allow for quantification of the true number of bacterial cells in the samples, which means that similar relative abundances in two samples can reflect very different amounts of bacterial biomass; some methods have recently been suggested to overcome this issue (Vandeputte et al., 2017). Even with these improvements, focusing on counting the abundances of microbial taxa tells very little about the activity of the community, and other methods could be far more informative (Cani, 2018). Finally, research has been mainly focused on bacteria, with far fewer publications regarding the archaeal, eukaryotic and viral members of the microbial communities, which tend to be more difficult to study. The 16S rRNA gene amplicon sequencing approach can be used for archaea, although it is complicated by the typically low amount of archaeal DNA in human samples, and the scarcity of archaeal sequences in 16S rRNA gene reference databases (Koskinen et al., 2017). For fungi, amplicon sequencing of the ITS region is useful, but also challenging, since this region varies so much between different fungal taxa that global sequence alignments are not possible (Nilsson et al., 2019). As for viruses, they are far too varied to be captured with any single marker, requiring more comprehensive approaches, such as shotgun metagenomics (Zou et al., 2016).

Considering all the pitfalls and limitations of the approach used in the studies of this thesis, it is clear that using additional methods will be required to fully understand the microbial community changes related to PD, and their potential relationship to disease pathogenesis and symptomatology. Shotgun metagenomic sequencing of all the DNA in fecal samples gives a more thorough picture of the different kingdoms present in subjects' gut communities and their functional capabilities. There could also be consistent shifts in the genetic repertoire of the community that are not reflected in the taxonomic composition, for example due to multiple, unrelated taxa being capable of performing the same functions. So far, there have been two metagenomic analyses of gut microbiota in PD. The small study by Bedarf et al. (2017), with 31 PD patients and 28 controls, all of them males, found a significant decrease in beta-glucuronate metabolism and a trend towards increased tryptophan metabolism. They also saw a decreased abundance of reads mapping to the "virus" category in PD patients; this reflects the number of viruses infecting archaea and bacteria, since the database used was specific to them. As the approach is DNA-based, it will not detect RNA viruses unless they are integrated in the host genome. Heintz-Buschart et al. (2018) used metagenomics specifically to identify some of the differentially abundant OTUs discovered in their amplicon analysis; interestingly, one of these taxa, which was depleted in PD patients, showed a high similarity to a *Melainabacterium* genome. These recently discovered relatives of *Cyanobacteria* (Di Rienzi et al., 2013) may be missing from older reference databases, therefore often going undetected in amplicon analyses. The example given by these two preliminary studies shows how the metagenomic approach could lead to new discoveries, and underlines the need for

more comprehensive analyses with larger study populations.

The shotgun metagenomics approach, although powerful, is still DNA-based, and will not reveal the actual activities of the community. For this, metatranscriptomic and metabolomic approaches are called for. Many of the PD and gut microbiota studies to date, including ours (I), have speculated about SCFAs as a moderator for the potential brain-gut link relevant to the disease (Bedarf et al., 2017; Heintz-Buschart et al., 2018; Hill-Burns et al., 2017; Keshavarzian et al., 2015; Li et al., 2017; Lin et al., 2018). So far, only one study has measured the levels of these metabolites in fecal samples, with 34 PD patients, 34 age-matched control subjects, and an additional 10 younger control subjects (Unger et al., 2016). They found that PD patients had significant decreases in absolute concentrations of acetate, propionate and butyrate, as well as in relative amount of butyrate, compared to age-matched controls (Unger et al., 2016). This seems to contradict a pioneering mouse study of PD and microbiota in which the presence of SCFAs seemed to aggravate neurological symptoms (Sampson et al., 2016). The effects of the different SCFAs may depend on their exact levels, and overall, the mechanisms of their influences on the human host remain unclear (Mulak, 2018). To clarify the situation in PD and to verify the findings by Unger et al. (2016), the SCFA levels of patients and control subjects should be further explored by larger independent studies. Additional analyses could be those targeting other molecules linked to the microbiome–gut–brain axis, including microbially produced neurotransmitters and hormones, and immune mediators such as cytokines. These comparisons should be performed in tandem with sequencing-based analyses, to look for associations between microbial shifts and the levels of the molecules of interest.

The various 'omic approaches tend to be costly, setting financial restrictions on the number of subjects and samples analyzed. A simpler solution better suited for larger cohorts could be to focus on the key taxa of interest discovered by the earlier 16S rRNA gene amplicon sequencing studies, such as *Akkermansia, Bifidobacterium, Lactobacillus* and *Prevotella*, and to confirm using a targeted approach, such as quantitative PCR (qPCR), whether the observed patterns hold true in different populations. The variation in the results of the different PD gut microbiota studies published so far makes one doubt the usefulness of microbiota as biomarkers for disease diagnostics. However, using uniform methodology could reveal a different picture. Larger study cohorts could also help in exploring the effects of various confounding factors. Particularly important would be to better characterize the interplay of the taxa of interest and the GI symptoms of PD, such as constipation (Fasano et al., 2015; Stirpe et al., 2016). Our analyses contrasting patients and controls have included corrections for constipation scores (I, IV), showing that our findings remain significant after taking these scores into account. Then again, our small comparison of microbiota in PD patients with and without IBS-like symptoms detected a decrease in *Prevotella* for the symptomatic patients (III); unfortunately we did not have enough control subjects with corresponding symptoms to compare their microbiota to the patients. The question of whether the microbiota changes seen in PD are only an epiphenomenon related to the GI symptoms, or relevant to the disease regardless of the subjects' specific non-motor symptoms, is difficult to answer conclusively, but comparing PD patients to subjects with similar GI symptoms in absence of parkinsonism would be a good starting point.

In addition to characterizing differences between PD patients and controls, a full understanding of the links between gut microbiota and PD should involve exploring whether there are further changes during the course of the disease. We performed some comparisons between patients with different degrees of disease progression in our follow-up study (IV), and one other study has attempted a disease progression analysis with a qPCR-based assay (Minato et al., 2017). Neither of these analyses found a strong difference between the

baseline and follow-up time points, and both showcase the key challenge of such an analysis, which is the lack of a standardized measure for disease progression. A two-year follow-up period is quite short, and a longer study, preferably a proper time series, could offer more insight into this topic. Similarly, to properly address questions of pathogenesis, one could envision a time series study following subjects with high disease risk or a prodromal disease to see how their microbial community evolves. Of course, such studies would be time-consuming and expensive, but there is only so much that can be said based on cross-sectional studies when it comes to questions of causal and mechanistic explanations. The other potential avenue for moving towards such answers is through animal experiments, for which there are some promising early results (Sampson et al., 2016). Unfortunately, the existing rodent models for PD leave much to be desired for (Francardo, 2018), and translating the results of rodent microbiota studies to humans is difficult due the many differences between these two organisms and their microbial communities (Nguyen et al., 2015). Optimally, future research would cover a combination of all the above-mentioned approaches – multiomic analyses, research focusing on specific microbial taxa of interest, animal modeling, and longitudinal observational human studies – to provide the full picture of PD and gut microbiota.

# 6 Conclusions

Exploring the microbial inhabitants of the human body in relation to Parkinson's disease using modern, DNA-sequencing based tools can offer new insight into the so far unresolved questions concerning disease pathogenesis and progression. In this thesis, I have presented the first examples of such analyses for gut, oral and nasal microbiota, as well as two additional gut microbiota analyses addressing the GI symptoms of the disease, and the temporal stability of the microbial communities two years after the initial sampling.

A question commonly asked in all studies such as the ones presented in this thesis is which specific microbial taxa differ between groups. An additional goal of the thesis was to evaluate the performance of the tools used for detecting such taxa. In line with past benchmarking studies, I found that the tools produced widely varying results, often with very little overlap. However, the key finding of our gut microbiota comparisons, the decreased abundance of *Prevotella* and *Prevotellaceae*, which has also been seen in several other studies of PD and gut microbiota, could be detected with all six tools used in the analyses presented in this thesis. This suggests that the strongest differences between groups can be discovered regardless of the method selected, and that using multiple tools can help pinpoint the most robust findings. In most cases, the differing taxa will not be as unambiguous, and the best course might be to report all potentially relevant results from multiple tools, waiting for future replication studies to clarify the situation. Of course, it will not be possible for any study to use every single tool out of the constantly growing selection, so further benchmarking studies to determine the best ones would be invaluable.

Two out of the three anatomic location studied in this thesis clearly warrant further research: the gut and the mouth. Our results regarding nasal microbiota were far less encouraging, with no differences in alpha or beta diversity, and no convincing signal for differences in the abundances of specific taxa. These results were supported by another recent study which found very few differences in nasal communities. While this does not entirely disprove the relevance of nasal microbes to PD, it does suggest that if there are any discoveries to be made, they will require a change of approach.

Regarding the oral and gut microbial communities, both were significantly different between PD patients and control subjects according to beta diversity analyses, and had many taxa that were over- or underrepresented in patients in confounder-corrected differential abundance comparisons. So far, no other studies regarding oral microbiota have been published; hopefully, this situation will change in the future, since they would be of interest to see if our findings can be replicated.

For the gut, several other research groups have published their findings after our pilot study, and all have found differences in beta diversity and the abundances of specific taxa between PD patients and control subjects. The detected taxa have been somewhat inconsistent between publications, possibly due to the different study populations and the variation in methodological details – among other things, few studies have chosen the same differential abundance detection tools. Our follow-up study, which included new samples from the subjects recruited for the pilot study collected on average 2.25 years later, showed differences in microbial communities that were similar to our original findings. We also attempted to look for disease progression related microbial signatures in patients, but the results were less

clear; this analysis was complicated by the clinical challenge of measuring disease progression.

Since there have already been several amplicon sequencing studies of gut microbiota and PD, future studies should preferably use other, complementary methods. We are currently in the process of performing a shotgun metagenomic sequencing analysis as well as several other 'omic analyses of our study subjects' samples. These will still be cross-sectional, single time point analyses. In addition to such comparisons, future studies should pursue longer follow-up periods with a proper time series sampling scheme. Additionally, targeted measurements of the few taxa that have been detected as differentially abundant by multiple studies, such as *Akkermansia, Bifidobacterium, Lactobacillus* and *Prevotella*, could help confirm whether these are truly consistent signals that could be useful in diagnostics, or that could potentially be manipulated as a future therapeutic approach.

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